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Studies in the Atomic Spectrometric Determination of Selenium, Mercury, and Rare Earth Elements

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STUDIES IN THE ATOMIC SPECTROMETRIC DETERMINATION OF SELENIUM, MERCURY, AND RARE EARTH ELEMENTS

A Dissertation Presented

by

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DEDICATION

To my husband, Dwight “D.J.” Harris, Jr.

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ABSTRACT

STUDIES IN THE ATOMIC SPECTROMETRIC DETERMINATION OF SELENIUM, MERCURY, AND RARE EARTH ELEMENTS

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The field of analytical chemistry is very important to today's society as more and more regulations and legislations emerge regarding trace elements in food, consumer products, medicines, and the environment. Like many areas of science, the current goals of trace elemental measurements and speciation are to increase knowledge on the subject and to improve upon current techniques by enhancing the figures of merit, such as accuracy and reproducibility, meanwhile balancing with the cost and time of analysis.

The topics covered in this work were investigated primarily through the use of inductively coupled plasma mass spectrometry (ICP-MS) and inductively coupled plasma optical emission spectrometry (ICP-OES). The phenomenon of compound-dependent responses in plasma spectrometry is discussed, seeking possible causes of it and offering some advice on how to prevent it. A new method was developed for the speciation of selenium in dietary supplements using anion-exchange chromatography ICP-MS. A novel method for the determination of total mercury at trace concentrations in rice was developed for use with conventional ICP-MS. Inductively coupled plasma mass spectrometry was also used for fingerprinting the rare earth elements in Maya archaeological pottery for provenance studies.

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CHAPTER 1

INTRODUCTION

According to the American Chemical Society,[1] "Analytical chemistry is the science of obtaining, processing, and communicating information about the composition and structure of matter. In other words, it is the art and science of determining what matter is and how much of it exists." Analytical chemists strive to provide the quantitative and qualitative information for many of life's major and minor endeavors, including the fields of medicine, pharmaceuticals, forensic science, food and environmental safety, petrochemicals, and even art and history, to name a few. For many of the problems encountered in these fields, the information needed is about the elemental composition or about the composition with respect to molecules containing the elements of interest.

Elemental analysis is only one aspect of the broader field of analytical chemistry, although there is nothing trivial about it. There is a very substantial research literature dealing with elemental analysis that continues to grow at the rate of many hundreds of original journal articles each year. Fortunately for practitioners in this field, the Royal Society of Chemistry has, since 1985, been publishing the *Atomic Spectrometry Updates*, which are annual reviews of the current literature on elemental speciation,[2] environmental analysis,[3] industrial analysis,[4] X-ray fluorescence spectrometry,[5] clinical and biological materials, foods and beverages,[6] and advances in atomic spectrometry.[7] These reviews detail the current challenges facing today's world as well as the new techniques developed to tackle these problems. Some of the major topics facing our society include

the detection of toxins, such as arsenic, lead, mercury, and thallium, in a variety of matrices, such as environmental waters, foodstuffs, children's toys, and human bodily fluids. Speciation analysis is also an important topic today; it is necessary not only to be able to detect a particular element such as arsenic or selenium, for example, but also to determine the chemical form(s) in which it is present. Because the toxicity of the element largely depends on its chemical form,[8] the ability to separate, identify, and quantify each species is of the utmost importance. This is of particular value when human exposure is concerned, as current legislations on maximum daily or weekly intake or exposure often do not take into account the different species and their relative toxicities.[8] This will perhaps change in the future as analytical techniques become more and more sophisticated and methods become standardized, allowing speciation data to be obtained routinely with confidence.

The determination of elemental concentrations within environmental or biological samples is not always a straightforward approach, particularly if the analytes of interest are present as trace elements within a complex material. Although the overall, problem-solving process may be similar, there is not one, universal method or technique by which all kinds of samples can be analyzed. The choice of analytical technique depends largely on the nature of the analyte, the sample matrix, the available instrumental techniques, instrumental capabilities and limitations, and what kind of information is desired from the results.

For example, the analysis of environmental water samples may be somewhat uncomplicated for instrumental techniques that require liquid samples, such as inductively coupled plasma mass spectrometry or inductively coupled plasma optical

emission spectrometry; however, the use of those same instruments for the determination of trace elements in solid samples requires a sample dissolution procedure to convert the material into a liquid matrix. Many issues can arise at this stage, as some solid matrices, such as geological samples, are difficult to dissolve without the use of dangerous reagents or lengthy, labor-intensive procedures.[9,10] Even so, incomplete sample dissolution may still lead to sample loss and poor analyte recovery from the original material.[10] Sample heterogeneity[11] can also lead to erratic results that perhaps can only be interpreted by careful statistical analysis. On the other hand, certain analytes, such as elemental mercury or dimethyldiselenide, are volatile and so they can be lost when high pressure or temperature digestions are used;[12] gentler dissolution techniques, such as extraction, might be employed in these cases, although they, too, tend to be labor-intensive and rather inefficient. Furthermore, when speciation information is desired, it is critical that the species do not undergo changes in their chemical structures during the sample preparation steps; species preservation, then, is a requirement of the selected sample dissolution technique, when these kinds of analyses are concerned.

Finally, the choice of instrumental technique, with all of its capabilities and limitations, must be considered. For example, the sample dissolution technique chosen for one's particular samples might be the most efficient there is, but this amounts to very little if the instrument is not compatible with measuring that final sample solution. Acid content, total dissolved solids, and interferences from other matrix components are some of the factors that could ruin an analysis, or even ruin the instrument components itself, in some cases. Most instrumentation requires calibration with standards, and so the utmost care is needed when building a calibration curve, as the analytical results can only be as

reliable as the calibration itself.[13] Further challenges may arise when an analytical instrument, originally believed to display compound-*independent* signal responses, is found to show a degree of dependence on the chemical form of the analyte.[14]

In plasma spectrometry, the choice of sample introduction system and of instrumental operating conditions and parameters also plays a significant role in the measurement of trace elements. The sample introduction system has been referred to as the "Achilles' Heel" of plasma spectrometry[15] and often is the major limiting factor of elemental analysis. For example, approximately only 1-3% of the sample is transported efficiently into the plasma,[16] whereas the rest of the sample is sent to waste. Furthermore, of the analytes within the sample that make it to the plasma, the fraction of those that are then excited or ionized depends on the excitation or ionization potential of the particular elements in question. Different sample introduction systems attempt to make improvements in the various processes and mechanisms involved with sample nebulization and transport, but none of them is capable of achieving 100% analyte transport and efficiency.

The work reported and discussed in this dissertation covers a range of topics within elemental analysis, including the speciation of selenium in dietary supplements, the determination of total trace mercury in rice, and the determination of multiple rare earth elements in archaeological pottery. All three of these trace elemental determinations require unique sample preparation and analysis techniques. An examination of the possible causes of, and how best to deal with, the phenomenon of compound-dependent responses in plasma spectrometry is included. Each topic is treated in a separate, stand-alone chapter, beginning with a critical review of the recent, relevant

literature. The particular challenges associated with each topic and the limitations of the current field of analytical chemistry are discussed, together with the experimental results of efficient, novel approaches by which these samples can be handled. The dissertation ends with a discussion of the remaining challenges associated with each of these topics, as well as some commentary on the future direction of analytical chemistry, as a whole.

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CHAPTER 2

COMPOUND-DEPENDENT RESPONSES IN PLASMA SPECTROMETRY

2.1 Introduction

2.1.1 The Compound-Dependent Response Phenomenon

Plasma source spectrometry is a useful technique for the determination of the elemental composition of liquid and gaseous samples, owing to its high atomization and ionization efficiency. Although inductively coupled plasma optical emission spectrometry (ICP-OES) and inductively coupled plasma mass spectrometry (ICP-MS) have different methods of detection, in both cases the analyte species are atomized to liberate individual atoms and the detector response should, therefore, be independent of the molecular formula of the initial substance.[1] Consequently, one might expect that a solution of sodium selenate with a concentration of 2 μg g^{-1} selenium (Se) would elicit the same detector response as a solution containing 2 μg g^{-1} Se in the form of selenomethionine. However, this does not always seem to be true. The differences in responses for different analyte species are most notable for arsenic and selenium. Some observers have found that some elements exhibit a *compound-dependent response*, in which the instrument produces a higher or lower signal intensity, depending on the element's speciation, i.e. the identity and configuration of the other atoms to which the analyte in question is bound. Furthermore, this same phenomenon appears with both ICP-OES and ICP-MS, which suggests that the differences in sensitivity arise because of processes that occur prior to the atomization step.[2]

Coupling high performance liquid chromatography (HPLC) to plasma spectrometry can sometimes make it more difficult to identify compound-dependent responses. Depending on the column used and choice of eluents, it is possible for some compounds to be partially retained on the stationary phase; the composition of the mobile phase may also affect the sensitivity because of effects related to carbon in the plasma. Only by calculating the mass balance after a chromatographic run can it be determined whether or not the compounds corresponding to the chromatographic peaks are exhibiting compound-dependent responses. The identification of compound-dependent responses in the chromatographic literature is complicated by the fact that researchers often do not include their calibrations with their final results. It is also important to note whether the researchers correlate peak area to the concentration of the *compound*, or if they correlate it with the concentration of the *element*. Finally, compound-dependent responses may be easier to recognize when looking at a chromatogram obtained by isocratic elution, as species with the same analyte concentration should possess similar peak areas, but gradient elution may make it less obvious at first glance, as peak shape can change with different flow rates or mobile phase composition. Only careful consideration of the chromatogram and its chromatographic conditions can truly identify the presence of this phenomenon.

The phenomenon of compound-dependent responses is a controversial one. If acknowledged at all, some researchers attribute it to minor variations in sample introduction systems, plasma conditions, matrix effects, and instrument designs. However, examination of past literature, particularly regarding selenium and arsenic,

reveals solid evidence of compound-dependency; the phenomenon is mentioned in only a small handful of these journal articles and even fewer contain explanations.

2.1.2 Controversial Evidence

Pan et al.[3] report finding major differences in Se sensitivities for the simultaneous separation of six Se and six As species in human urine by ion-pair- reversed-phase liquid chromatography-ICP-MS. Although the As species showed no significant signs of compound-dependency, the sensitivities of the organic Se species (selenomethionine, selenourea, selenoethionine, selenocystine, and trimethylselenonium iodide) were between 59-79% of the sensitivity of sodium selenite. The researchers proposed that differences in atomization and ionization efficiencies were responsible. A listing of the most relevant Se compounds and their structures may be viewed in the Appendix.

Narukawa et al.[4] investigated the different sensitivities of As^{III} versus As^{V} using ICP-MS, ICP-OES, and ICP-sector field-MS. Whereas As^{III} consistently demonstrated lower sensitivity than As^{V}, the researchers found no such difference between Se^{IV} and Se^{VI}, which share many properties with the corresponding As species. The researchers propose that the arsenic species, after complete atomization, recombine within the plasma to form hydrogenated species, thereby changing the species' sensitivities in an unpredictable way; they also showed that the effect was dependent upon the distance between the sample introduction system and torch, although the formation of these hydrogenated species and the mechanism by which they might alter the sensitivity are still not understood.

Differences in sensitivity have also been documented when using ultrasonic nebulizers,[5-7] pneumatic nebulizers,[2,3] and even direct injection nebulizers,[8] and so differences in technique and analyte loss due to interactions with a spray chamber are not likely causes. For example, Creed et al.[5] saw a similar effect; the sensitivity of As^{III} by ion chromatography-ICP-MS with an ultrasonic nebulizer (USN) was 70-80% of that of As^{V}. The signal difference was attributed not to matrix or oxidation state effects, but rather to differences in desolvation within the USN, which is unlike the desolvation processes of traditional pneumatic nebulizers. According to Narukawa et al.,[4] the distance between torch and sample introduction is increased for an ultrasonic nebulizer, leading to greater differences in As sensitivity. The desolvation effect from an USN cannot be the only effect in play here, however, as Yu et al.[2] also demonstrated a similar depression of the As^{III} signal for traditional pneumatic nebulizers with ICP-OES and ICP-MS. A bias of 3% between the measured spectrometric values and gravimetric preparation values was observed when measuring SRM 3103a Arsenic Spectrometric Solution, and further investigation by HPLC-ICP-MS confirmed not only that both As^{III} and As^{V} were present in the material, but also that the As^{III} yielded a response that was typically 8% lower than the As^{V} signal with ICP-OES. The researchers admit that the cause of this "speciation effect" requires further investigation, but they offer that it may be related to differences in aerosol transport, droplet desolvation, or atomization.[2]

In contrast, Gammelgaard et al.[6,9] report no such sensitivity differences for selenium species when using pneumatic nebulization, but signal enhancement was seen for selenate and trimethylselenonium iodide when using an USN. Solvent and instrument

parameters were investigated, and it was shown that pH and the temperature of the USN influenced the magnitude of the sensitivity difference.[6]

Several other studies exist, demonstrating lower sensitivity for As^{III} or Se^{IV}. Using ultrasonic nebulization, Budič[7] offers reduced transport efficiency as a possible explanation while showing that the differences are exacerbated in the presence of high salt concentrations; Yang et al.[10] report, for ICP-OES with thermospray sample introduction, a lower sensitivity for selenite, which became even more significant with the addition of methanol. They suspected that the selenite was being reduced to elemental Se, which then was lost to the sample introduction system. Interestingly, Larsen and Stürup[11] found, in an investigation into signal enhancement in the presence of carbon, a slightly different trend using pneumatic nebulization ICP-MS; in this study, As^{III} exhibited the greatest sensitivity, whereas As^{V} was only 93% and 6 different organo-arsenic species ranged from 67-88% of the As^{III} signal. Alternatively, differences in volatility were named as a possible explanation by Juresa et al.,[12] after the volatile selenium species, dimethylselenide (DMSe) and dimethyldiselenide (DMDSe), produced up to 58-fold higher responses than selenite.

Preliminary investigations of compound-dependent responses in plasma spectrometry were made by Dodova.[13] Several theories and their likelihood were discussed. Of the species involved [selenite, selenite, selenious acid, selenomethionine, selenocystine, and seleno(methylseleno)cysteine], all of the inorganic species exhibited similar calibration slopes, whereas the organic species reproducibly exhibited significantly lower responses by both ICP-OES and dynamic reaction cell-ICP-MS. Sulfur (S) compounds were also measured, but did not show much variation between

organic and inorganic species. The addition of germanium and tellurium internal standards made no difference, nor did increasing plasma robustness (raising RF power and decreasing nebulizer gas flow). Also dismissed was the idea that the more volatile species could be lost by sticking to the walls of the spray chamber or peristaltic pump tubing, as washout profiles proved to be similar to those of known, unretained analytes. Furthermore, the presence of carbon enhancement and easily ionizable element effects were considered an unlikely cause, as the same relative slopes were observed for solutions prepared in aqueous media versus those prepared in mobile phase containing methanol and phosphate buffer. By comparing bond strengths, it was also discussed, although not proven, that the cause of compound-dependency is likely not related to the formation of oxide ions as a result of incomplete atomization or recombination within the plasma.

While the researchers acknowledge the existence of compound-dependent responses, and some even attempt to offer an explanation, there are many other examples in the published literature in which no such acknowledgments are made. For instance, Peachey et al.[14] show mixed-mode HPLC-ICP-MS chromatograms by isocratic elution that also demonstrate very different peak areas for the seven Se species measured, although it is unclear if the peaks correspond to 10 ng g^{-1} of the selenocompound, or 10 ng g^{-1} of Se present as those seven different species. Seven selenocompounds, each present as the same concentration of Se, were separated by Goessler et al.[15] using strong cation-exchange HPLC with ICP-MS detection, and although retention time is discussed in great detail according to pH, no mention of the differences in peak areas is made. Finally, another example that shows evidence of compound-dependency is in a study by

Brennan et al.[8] Five different species of arsenic were separated by nano-HPLC using isocratic elution with ICP-MS detection and a direct injection nebulizer. Of the species separated, arsenic in the form of sodium arsenite exhibited the greatest signal, while dimethylarsenic acid, disodium methylarsonic acid, sodium arsenate, and *p*-arsanilic acid exhibited signals that were 60%, 43%, 43%, and 48% relative to the arsenite signal, respectively. Again, no mention is made of this seemingly strange phenomenon.

2.1.3 Implications

As evidenced in the literature survey in section 2.1.2, compound-dependent responses exist and clearly show discrepancies in response factors between compounds of equal concentrations. Ignorance of this phenomenon can quite possibly lead to inaccurate quantification. Just as it is important to match the matrix of calibration standards to the matrix of one's samples, so too might it be necessary to calibrate using the same species as found within the samples of interest. For example, if one's samples contain primarily selenomethionine, then the most accurate way of quantifying them would be to calibrate with a selenomethionine standard. Calibrating with standards prepared from sodium selenate, on the other hand, would likely produce a small, but significant, bias in the results, given the different response factors of the two compounds.

2.1.4 Possible Explanations

Several theories have been proposed as to the possible explanation for the occurrence of compound-dependent responses in plasma spectrometry, many of which have been tested and ruled out by Dodova.[13] Firstly, limitations inherent to ICP-MS,

such as isobaric interferences and polyatomic ions, have been discarded because the same effects are observed both in DRC and standard mode, as well as in ICP-OES.[13] Aside from that, the remaining possibilities have been organized into three major categories for the purpose of this survey, all of which are interrelated: the chemical/physical behavior of these compounds and how they interact with other molecules, plasma operating conditions, and issues arising from different sample introduction systems.

2.1.4.1 Chemical and Physical Behavior

The first category, compound-dependent responses arising from the chemical and physical properties of the compounds in question, and how they interact with other molecules within the matrix, includes a long list of possibilities. For instance, it is well known that the presence of 1-3% carbon in solution can increase the signal of elements whose first ionization energies are 9-11 eV, which includes Se, S, and As, but it is also documented that carbon content exceeding 3% can actually decrease signal due to lowering of the plasma's temperature.[13] This could suggest that the addition of carbon generates a greater increase in inorganic species' signal, than it does in organospecies. Dodova demonstrated that organoselenium responses were consistently lower than the responses of inorganic selenium species, however, regardless of the presence of carbon in the matrix.[13] On the other hand, though, Larsen et al.[11] observed a greater increase in selenomethionine signal than in selenite's signal when increasing quantities of MeOH were added, before they both tapered off. The researchers attributed this effect to an increased population of carbon atoms in the plasma, which increases the ionization of elements that have ionization energies lower than that of carbon.

Similar to the signal enhancement caused by the presence of carbon in the matrix, so, too, can the signal be altered in ICP-OES by the presence of easily ionized elements, in a process termed the easily ionized element (EIE) effect. Easily ionizable elements such as sodium (Na), potassium (K), and calcium (Ca) produce an abundance of electrons in the plasma because of their low ionization potentials, which can then either collisionally ionize certain elements (thereby suppressing atomic emission and enhancing ionic emission) or recombine with other elements (thereby enhancing atomic emission and suppressing ionic emission), although typically this effect is not seen until the EIE is present at a concentration of approximately 1000 mg L^{-1}.[16] The EIE effect can, however, be diminished by operating under robust plasma conditions. Furthermore, the presence of Na in certain inorganic Se species cannot quite explain the difference in signal intensity; for instance, a 10 mg L^{-1} solution of sodium selenite in an aqueous matrix has approximately 5 mg L^{-1} Se, and only 3 mg L^{-1} Na, a quantity not sufficient enough to exhibit a noticeable EIE effect.

Besides effects from carbon and EIEs, other matrix effects could be caused by differences in species' response, although Dodova[13] showed that the use of internal standardization made no difference to the observed trends.

Other possible explanations of the physical or chemical nature could include the differences in volatility, desolvation, vaporization, aerosol transport, or atomization of these individual species.[2,5] For instance, if the organoselenium species atomize in the plasma earlier than the inorganic species, then these organospecies could be lost to lateral diffusion, thereby diminishing the amount that reaches the detector and making it appear that the inorganic species produce greater signals. On the other hand, the droplet size

distribution or even surface properties of those droplets could potentially vary between species, which may attribute to this phenomenon. It is also possible that some of these species could be forming refractory oxides,[13,17] hydrides,[4] or even colloidal species within or prior to the plasma, which may then also lead to further differences in desolvation and atomization mentioned above.

Finally, selenium's oxidation state within each of these different selenocompounds may play a role in its differences in instrument response. Although, as mentioned earlier, Creed et al.[5] considered that the difference in signal between As^{III} and As^{V} was not a result of the different oxidation states, but rather because of loss of the As^{III} species by the USN's desolvation system, Yu, et al.[2] demonstrated that something else could be participating in the effect, as the same trend was observed with traditional pneumatic nebulization. Therefore, the difference in oxidation states should not be entirely ruled out as a possibility.

2.1.4.2 Plasma Operating Conditions

The second category proposed is that compound-dependent responses might arise from the different plasma conditions and processes within it. Temperature, for instance, has a large effect on responses in plasma spectrometry. It is well known that there are a number of zones within the plasma, and that the temperature across these zones is not uniform.[18] Typically, the greatest amount of excitation or ionization occurs in the regions of higher temperature and will consequently give rise to a higher signal (depending on ICP-OES or ICP-MS, respectively).[18] Unlike most analytes that are atomized in the central channel of the plasma, selenium exhibits a very distinct excitation profile in

optical emission spectrometry, in which the greatest amount of signal is generated off-center, while the central channel, itself, yields a low signal-to-noise ratio.[16,17] Many other factors can influence the temperature of the central channel, such as nebulizer gas flow rate and aerosol transport, RF power, and, as mentioned earlier, the presence of carbon, to name a few. With ICP-OES, a good indication of plasma robustness is to monitor the temperature via the MgII/MgI intensity ratio, since the higher the temperature of the plasma, the more likely the ionic line (Mg 280 nm, or MgII) will be excited, relative to the atomic line (Mg 285 nm, or MgI).[19]

Torch viewing height, or the distance between the tip of the injector and the load coil, can also affect the analytical signal, as they can influence where analytes are atomized in the plasma and how much time they spend in the plasma. Increasing the length of time analytes spend in the plasma decreases the occurrence of refractory oxide formation,[13] although can also encourage more collisions, recombination, and lateral diffusion. This, however, is only possible with ICP-OES, as the torch viewing height and sampling depths are fixed in ICP-MS.[4] As mentioned earlier, Narukawa et al.[4] hypothesize that sampling depth, leading to hydride formation, is the reason why As^{III} and As^{V} produced such variable signals, although they found no such variation for Se^{IV} and Se^{VI}; as sampling depth was kept at the shortest possible distance, As^{III} exhibited a higher signal than As^{V}, but this trend reversed itself when the distance was elongated.

2.1.4.3 Differences in Sample Introduction Systems

The final category of possible effects includes the sample introduction system as the cause of the discrepancies in instrument response factors. Different types of

nebulizers were considered, although, as mentioned in section 2.1.2, compound-dependent responses were observed for both ultrasonic and pneumatic systems.[2,5] The wide range of droplet sizes produced by traditional nebulizers could also be a possible factor; however, USNs typically produce a much more uniform droplet size and were still found to exhibit signs of compound-dependency.[2] As mentioned above, the sampling depth can vary between different sample introduction systems, potentially causing changes in analyte signal for the different compounds analyzed.[4] Even removing the sample introduction system from the equation by using a direct injection nebulizer (DIN) has been found to produce variation in signal with different species;[8] therefore, potential sample loss from sticking to the walls of the spray chamber also cannot be considered a source of compound-dependent responses.

2.2 Research Overview

The phenomenon of compound-dependent responses in plasma spectrometry was investigated here. Calibration solutions were prepared and analyzed via ICP-OES and ICP-MS to confirm that the same instrumental responses were observed as seen by Dodova.[13] Rather than repeating the work of Dodova,[13] new theories were tested in an effort to more fully understand this phenomenon.

Whereas Dodova[13] investigated As, Se, and S compounds, in the work described here, only Se compounds were investigated. More attention was given to experiments performed by ICP-OES because the instrumental technique has the benefit of having more parameters and conditions under the control of the operator, compared to the operating parameters of the available ICP-MS instrument. In addition to testing the same

selenocompounds previously examined, namely sodium selenate, sodium selenite, selenomethionine, selenocystine, selenious acis, and seleno(methylseleno)cysteine, this work also included results for a few more selenium-containing species on which to build and test more hypotheses: sodium selenide, elemental Se, zinc selenide, cupric selenate, and dimethyldiselenide. In addition to using statistical analysis to compare calibration curves for each of these compounds, changes in sample introduction, sampling depth, torch position, and viewing position (axial versus radial) were made. The temperature of the plasma was monitored as a function of these different species, and droplet size and surface properties were examined in an effort to better understand the cause of compound-dependent responses.

2.3 Experimental

2.3.1 Instrumentation

Instruments used were a PerkinElmer SCIEX (Ontario, Canada) ELAN 6100 plasma-source mass spectrometer and PerkinElmer, Inc. (Shelton, CT) Optima 4300 and 8300 flat plate optical emission spectrometers. The plasma mass spectrometer was equipped with a PerkinElmer, Inc. cross-flow nebulizer and Scott spray chamber, while the 4300 and 8300 emission instruments utilized a GemCone nebulizer with glass cyclonic spray chamber and an experimental nebulizer and spray chamber, respectively, also from PerkinElmer, Inc. For some experiments, a T-junction connector was added to the GemCone nebulizer in order to flow sample and a magnesium solution into the instrument, simultaneously for online addition. A CEM Corporation (Matthews, NC) MARSXpress microwave system, Model 230/6 with

Teflon vessels was used for selenium oxidation. Instrumental conditions and other experimental parameters may be viewed in Table 2.1.

2.3.2 Reagents and Sample Materials

All solutions were prepared using 18 MΩ cm deionized (DI) water from a Barnstead E-pure system (Bedford, MA). Hydrogen peroxide, certified ACS plus nitric acid, hydrochloric acid, and cupric selenate ($CuSeO_4$) were obtained from Fisher Scientific (Fairlawn, NJ). Sodium hydroxide solution (25% w/v) was purchased from Reagents, Inc. (Charlotte, NC). Seleno-DL-cystine ($(SeCys)_2$), seleno(methyl)selenocysteine hydrochloride (SMSC), seleno-DL-methionine (SeMet), sodium selenate decahydrate 99% (Se^{VI}), sodium selenite 99% (Se^{IV}), and selenious acid 98% (H_2SeO_3) were all obtained from Sigma-Aldrich Co. (St. Louis, MO). Sodium selenide (Na_2Se) was purchased from both Pfaltz & Bauer, Inc. (Waterbury, CT) and Sigma-Aldrich Co., while zinc selenide 99.99% metals basis and elemental Se (Se^0), supplied as selenium powder, was from Strem Chemicals (Newburyport, MA). Triton X-100 was from Aldrich Chemical Company, Inc. (Milwaukee, WI) and the magnesium atomic spectroscopy standard came from PerkinElmer, Inc. (Shelton, CT). To distinguish the two sources of Na_2Se, the one from Sigma-Aldrich Co. was designated as Na_2Se (SA), whereas the one from Pfaltz & Bauer, Inc., was designated as Na_2Se (PB).

2.3.3 Analytical Procedures

2.3.3.1 Preparation of Standard Solutions

Stock solutions were prepared by dissolving approximately 10-150 mg of the Se compounds (Se^{IV}, Se^{VI}, H_2SeO_3, Na_2Se, SeMet, SMSC, $(SeCys)_2$, and $CuSeO_4$) in DI water, with the exception of $(SeCys)_2$, Se^0, and ZnSe which were first dissolved in approximately 1 mL HCl (5%), 1 mL HNO_3, and 0.5 mL HNO_3, respectively, before dilution with DI water. Dimethyldiselenide was also dissolved in 1 mL HNO_3 prior to dilution. All stock solutions were clear and colorless, with the exception of Na_2Se (SA), which changed from clear to pale pink, and finally to a dark orange-red color within a few minutes. The preparation of these stock solutions was performed periodically, to ensure proper sampling. From these stocks, calibration standards were prepared by diluting further with DI water to approximately 10 µg g^{-1} Se for analysis by axial-viewed ICP-OES, 100 µg g^{-1} for radial-viewed ICP-OES, or to 10 ng g^{-1} Se for analysis by ICP-MS. The solutions were stored up to several months in the refrigerator without degradation.

2.3.3.2 Calibration Slopes for ICP-OES and ICP-MS

Instrumentation was optimized according to standard protocols, i.e. the emission spectrometer's viewing position was aligned using a standard of Mn, and nebulizer flow rate was determined experimentally based on maximum signal intensity, whereas the mass spectrometer was optimized according to maximum indium signal and optimal nebulizer flow rate was determined by the instrument software. Both instruments were set to high RF power to ensure robust plasma conditions. Each of the selenium standards

prepared according to section 2.3.3.1 was measured along with a blank solution in order to create a two-point calibration for each species. It was assumed that these solutions would follow linear functions. This same procedure was used as the basis for subsequent experiments discussed in sections 2.3.3.3 through 2.3.3.8. Compound-dependent responses were found to be similar when using both ICP-OES (axial and radial viewing mode) and ICP-MS, and so the majority of experiments were performed using axial-viewed ICP-OES, as the emission instrument allowed for more control over sample introduction parameters and made it possible to monitor plasma temperature through Mg emission intensity.

2.3.3.3 Axial Viewing Position in ICP-OES

It is known that Se, when viewed in both axial and radial mode ICP-OES, exhibits an unusual spatial profile, where the maximum emission intensity is not observed in the center of the plasma, but rather it is distributed about an annular shape with two asymmetric maxima on either side of the central channel.[18,20] This is because the central channel is typically cooler in temperature than the zones surrounding it due to different atom distributions.[21,22] Therefore, if the instrument is aligned to Mn 257 nm, which will yield the maximum intensity near the center of the plasma because of its low excitation energy, then subsequent readings for Se solutions would be monitoring signal from the depression between the two maxima, as Se is more difficult to excite.[21] Increasing plasma robustness by lowering the flow rate of nebulizer gas can lessen the severity of the dip between the two maxima because the lower flow will increase analyte residence time in the plasma, thereby increasing the extent of vaporization, atomization, ionization,

and excitation.[21] It was theorized that, by varying the axial viewing position and changing nebulizer flow rate, one could find out if these different Se species might exhibit slightly different emission profiles, to explain why some yield higher responses than others. In order to do this, each Se solution was aspirated continuously, running calibration after calibration while varying the viewing position from the far left-most region of the plasma to the far right. This was repeated with several different nebulizer flow rates.

2.3.3.4 Torch Position in ICP-OES

The torch position was adjusted by changing the distance between the tip of the injector and the RF coil. This was to see if the distance would augment or lessen the extent of compound-dependent responses for each of the Se solutions, as this could influence where in the plasma certain species are atomized and how long of a residence time they experience, which could in turn affect the extent of dissociation, ionization, and recombination processes.

2.3.3.5 Temperature and Robustness of Plasma

According to Mermet,[23] robust plasma conditions in ICP-OES can be created by raising the RF power and lowering the nebulizer gas flow; while different matrices can lead to interference effects, robust conditions help to keep these effects to a minimum, regardless of the analytical line.[24] These robust conditions help limit aerosol loading of the plasma and matrix interferences while increasing temperature and electron density.[21,25] Monitoring the intensity ratio of Mg^{II}/Mg^{I} can be used as a diagnostic for

departure from local thermodynamic equilibrium in the plasma;[19] the higher the ratio, the more likely that species within the plasma approach optimum atomization and ionization processes, and therefore, a higher plasma temperature and robustness may be assumed.

To see if plasma temperature and robustness were altered as a direct result of the particular Se species being introduced, the Mg^{II}/Mg^{I} intensity ratio was monitored simultaneously along with the Se standards. Rather than spiking each standard with Mg, which could introduce a small amount of error into the comparison, instead, a 1.0 μg g^{-1} Mg standard was prepared and run by online addition via a T-junction; this way, the Mg standard would mix with the Se standard prior to nebulization, and the Mg concentration could be kept constant for each different Se standard analyzed.

Before beginning the online addition experiment, the same Mg standard solution was run for two hours beginning at instrument start-up in order to establish an idea of how much the ratio fluctuated in the absence of Se.

The sensitivities of all twelve Se standards, both with and without the addition of 0.1% HNO_3, were compared while nebulizing at the optimal nebulizer flow rate of 0.55 L min^{-1} (determined experimentally) versus a non-optimized flow rate of 0.70 L min^{-1}.

2.3.3.6 Droplet Properties

2.3.3.6.1 Addition of Surfactant

The addition of surfactant to liquid samples has been known to change the physical properties of the solution, making it possible for greater efficiency as well as a greater production of volatile species.[26] If compound-dependency were related to the

difference in aerosol droplet formation and behavior as a result of these different physical properties, then it was thought that adding surfactant to each of the solutions might alter the surfaces of these droplets, making them behave more similarly. To test this theory, the Se standards were measured before and after the addition of 0.1% Triton X-100 in order to make a direct comparison between calibration slopes.

2.3.3.6.2 Droplet Size Distribution

Differences in droplet size distribution between the different selenium species is another possible reason that could bring about different sensitivities in ICP spectrometry. Although ultrasonic nebulization has been known to produce smaller droplet sizes,[6] it is also subject to other issues such as desolvation problems;[6,7] furthermore, it has already been shown that compound-dependent responses are observed in studies performed using ultrasonic nebulization.[5-7]

In order to obtain smaller and more uniform droplet sizes within the primary aerosol without the desolvation issues arising from ultrasonic nebulization, an experimental design nebulizer from PerkinElmer, Inc. was used. The prototype consisted of a vibrating mesh,[27,28] capable of producing uniform droplets between 6 and 10 µm in diameter and achieving over 90% transport efficiency, whereas traditional pneumatic nebulizers typically produce droplets in the range of sub-µm to 30 µm diameters, with only 1-3% transport efficiency at a normal pump flow rate of 1.0 mL min^{-1}.[28] The prototype's aerosol generation is independent of the nebulizer gas flow[27,28] and is only limited by how much can be introduced before the plasma is overloaded, much like a direct injection nebulizer. Furthermore, the prototype does

not have a traditional spray chamber,[27] but rather the aerosol droplets are delivered directly to the base of the injector, thereby removing the potential for analyte loss due to collisions or coagulation.

The 10 µg g^{-1} Se standard solutions were measured using the PerkinElmer experimental nebulizer under the conditions listed in Table 2.1. The Se standards were prepared in 0.1% HNO_3 to prevent wetting of the mesh face that could block the aerosol production.

2.3.3.6.3 Aerosol Ionic Redistribution

The aerosol ionic redistribution (AIR) effect, in which the process of nebulization can produce a net charge on the droplets, resulting in an aerosol with very different surface properties from that of the bulk solution,[29] has been documented in atomic emission spectrometry.[29-31] Besides producing the potential for large differences in droplet size distribution, this net charge could also affect the droplet's behavior in terms of aerosol transport, interference effects, and consequently emission profiles, as enrichment of analyte ions within the aerosol may occur.[30] We proposed that the act of nebulization could be changing the surface properties of the individual droplets, thereby causing a different response within the plasma that varies according to selenium species nebulized.

The drainage tube coming out of the emission spectrometer's cyclonic spray chamber was redirected into a beaker, so as to collect the nebulized solution that did not make it into the spectrometer. This collected waste for each Se species was then nebulized once more into the instrument and repeated for a total of three times, in an

effort to determine if any analyte ion enrichment could be occurring with each subsequent step. It was theorized that the sensitivity of the fraction of selenate that goes to the spectrometer would be equivalent to the fraction that goes to waste, whereas it was expected that the bulk solution of Na_2Se (PB) going to waste might be more enriched in Se than the fraction that makes it into the instrument, thereby resulting in a lower sensitivity for that compound.

2.3.3.7 Oxidation States of the Species

Another theory to explain why different sensitivities might be observed for different Se species is that the different oxidation states may be playing a role. Sodium selenate, which usually demonstrates the greatest sensitivity, contains Se in the +6 oxidation state; therefore, it was hypothesized that, by oxidizing all of the compounds to the +6 state, the other selenium species might then yield sensitivities equivalent to that of Se^{VI}.

The microwave digestion procedure utilized 0.5 mL HNO_3 and 1 mL H_2O_2, added to the vessels containing 50 mg of sample. These vessels were microwave digested at 1600 W at 100% power, ramping for 20 min to 180 °C and holding for 40 min before cool down. The digested material was diluted to 50 g with DI water, and the appropriate aliquot mass was further taken and diluted once more to 10 g, so that the final concentration was around 10 μg g^{-1} Se. All solutions were clear and completely digested.

2.3.3.8 Colloidal Species

A theory to explain why Na_2Se (PB) might be displaying such lower sensitivity is that it could be forming a colloidal solution. Modifying the procedure from Stroyuk et al.,[32] colloidal Se was prepared by mixing elemental Se (800 mg) and sodium sulfite (5 g) together in hot water (50 mL) to make a solution of Na_2SeSO_3, which was very dark-purple in color. This solution was then refluxed with HCl to produce a "hot"-pink solution of colloidal Se, which turned clear after about 2 h of refluxing. Whereas Stroyuk et al. also added 10% gelatin to the solution, this step was omitted in order to spray the solution into the emission spectrometer. The final solution was diluted with DI water to 10 µg g^{-1}. It was expected that, if the colloidal Se solution should produce a signal approximately 50% of that of selenate, then it could be concluded that Na_2Se (PA), too, must also be forming a colloidal solution, limiting its sensitivity in the plasma.

2.3.3.9 Data Analysis

All results were processed using Microsoft Excel 2007. Slopes were compared by statistical analysis according to a modified t-test,[33,34] illustrated in Equations 1-5. The average intensity of ten replicate measurements was used to calculate the slopes, and the standard deviation of these replicates provided sufficient data for statistical analysis, where H refers to the higher of the two standards and L refers to the lower of the two standards, i.e. the blank. Standard concentration is indicated by X, $\bar{y}$ is the average intensity, M is the number of replicates, s is the sample standard deviation of the noise at X, and s_{pooled} is the pooled standard deviation. The calibration lines are assumed to be

homoscedastic and Gaussian with white noise, and the critical t value was based on two-tailed probability at the 95% confidence.

$$slope_1 \equiv \frac{\bar{y}_{H1} - \bar{y}_{L1}}{X_H - X_L} \quad \text{and} \quad slope_2 \equiv \frac{\bar{y}_{H2} - \bar{y}_{L2}}{X_H - X_L} \tag{1}$$

$$s^2_{pooled,1} \equiv \frac{(M_{XH} - 1)s^2_{H1} + (M_{XL} - 1)s^2_{L1}}{M_{XH} - 1 + M_{XL} - 1} \frac{1}{(X_H - X_L)^2} \tag{2}$$

$$s^2_{pooled,2} \equiv \frac{(M_{XH} - 1)s^2_{H2} + (M_{XL} - 1)s^2_{L2}}{M_{XH} - 1 + M_{XL} - 1} \frac{1}{(X_H - X_L)^2} \tag{3}$$

$$t \equiv \frac{slope_1 - slope_2}{(s^2_{pooled,1} + s^2_{pooled,2})^{1/2}} \tag{4}$$

$$\text{total degrees of freedom} = 2(M_{XH} - 1) + 2(M_{XL} - 1) \tag{5}$$

2.4 Results and Discussion

2.4.1 Calibration Slopes for ICP-OES and ICP-MS

The average slopes are shown in Table 2.2 for the twelve Se standards by ICP-OES (a) and ICP-MS (b), respectively. The percentages shown refer to the ratio percent of each Se compound's slope in relation to the slope of Se^{VI}. Statistical analysis of the results by ICP-OES shows that the slope of Se^{VI} was not significantly different from Se^{IV}, H_2SeO_3, or $CuSeO_4$, but was to all of the other compounds. The slopes of H_2SeO_3, $CuSeO_4$, ZnSe, $(SeCys)_2$, and Se^0 were also determined not to be significantly different from one another. On the other hand, SeMet and SMSC were found to be statistically different from all slopes except for each other, while the slopes of Na_2Se and DMDSe, separately, were significantly different from all species measured.

Previously, it was thought that the inorganic species tended to exhibit greater signals than the organic species,[13] but having added a few more Se species into the

suite of standards revealed that this might not be the case, as Se^0, ZnSe, and $CuSeO_4$ exhibited similar sensitivities to the organic species. Concerning the two Na_2Se solutions, Na_2Se (PB) produced the lowest signal of all, being roughly half the response of Se^{VI}, while Na_2Se (SA) produced the largest signal of all. Furthermore, the measurement of a volatile species, DMDSe, revealed a still slightly low response, which is the opposite of what Juresa et al.[12] found, and so the theory of species' volatility is likely not at play here.

Over the course of two years, some variation of these responses were seen, but in most cases, compound-dependency was still observed; typical sensitivities, relative to sodium selenate, were between 98-100% for sodium selenite, 97-102% for selenious acid, 89-98% for selenomethionine, 92-97% for seleno(methylseleno)cysteine, 93-99% for selenocystine, between 91-100% for elemental Se, 47-51% for sodium selenide from Pfaltz & Bauer, 105-111% for sodium selenide from Sigma-Aldrich, 95-98% for zinc selenide, 96-100% for cupric selenate, and finally, 87-95% for dimethyldiselenide.

2.4.2 Axial Viewing Position in ICP-OES

Results of varying axial viewing position and nebulizer gas flow rates for Se^{VI} are shown in Fig. 2.8.1. As expected, the Se emission profile exhibited two maxima, one around 16 mm, and a slightly higher one at 14 mm (with the entire horizontal position being from 0 to 30 mm), although this was consistent for each different species. Also as expected, as the nebulizer flow was decreased, the minimum between the two peaks began to become less pronounced as more photons were

produced in that region. This minimum nearly reversed itself once the flow rate was set to 0.48 L min^{-1}, although the overall intensity was low because less of the sample was being delivered to the plasma. Despite varying the viewing position and nebulizer flow rate, it was apparent that compound-dependency was still present, as each different Se species exhibited almost exactly the same profile while the sensitivities varied. As such, Figure 2.1 only shows the profile for Se^{VI}, but the observed maximum intensities were approximately 18,000 cps for Se^{VI}, 17,000 for SeMet, and around 9,000 for Na_2Se (PB). Although the shape of the profiles between the different species did not vary, this experiment proved that the compound-dependent responses must be arising prior to the atomization stage, as the distributions in the plasma are the same. From the graphs, it was determined that a nebulizer flow rate of 0.55 L min^{-1} was optimum.

2.4.3 Torch Position in ICP-OES

The results of varying the torch position in Figure 2.2 indicate that the height made no difference in the observed compound-dependency. While increasing the distance between injector tip and load coil raised the signals of all Se compounds measured, it did so proportionally, so that Na_2Se (PB) was still roughly 50% of the Se^{VI} signal regardless of the distance between torch and load coil.

2.4.4 Temperature and Robustness of Plasma

The results of running the Mg standard solution for two hours after instrument start-up in the absence of Se, though not shown, demonstrated a very slight increase

of about 1% in the Mg^{II}/Mg^{I} intensity ratio over the course of the analysis, 0.5% of which was most likely due to instrument warm-up during the first 20 min. after ignition. Therefore, it was concluded that the Mg^{II}/Mg^{I} intensity ratio was stable enough to be used as a diagnostic tool for plasma temperature and robustness.

The same Mg standard solution was run continuously throughout the entire course of the experiment, changing only the Se standard solution it was mixed with at the T-junction prior to nebulization. A blank solution of pure DI water was run three separate times throughout the experiment as a means of quality control to verify that the Se solutions were washing out completely and that the same Mg ratio was observed for each blank.

Figure 2.3 shows the result of this experiment. Interestingly, opposite trends were observed, depending on the nebulizer flow rate used. For instance, Figure 2.3a shows the optimized nebulizer flow rate of 0.55 L min^{-1} for the solutions without acid. The plasma, during aspiration of the three Na-containing species, had higher Mg intensity ratios than when aspirated with the rest of the compounds. The plasma, when aspirated with the remaining compounds without Na, exhibited similar ratios equivalent to the blank. A nearly identical trend was observed for the solutions containing 0.1% HNO_3, run at the same nebulizer flow rate (not shown). Figure 2.3b shows the non-optimized nebulizer flow rate of 0.70 L min^{-1} and the solutions prepared without acid; in this figure, the opposite trend is observed, in which now the plasma, during the aspiration of the three Na-containing species, had lower Mg ratios than with the rest of the species. Again, the solutions containing acid measured at this flow rate were nearly identical to the non-acidified solutions. Also of note was

the disparity between the two different solutions of Na_2Se. Furthermore, the Mg intensity ratios in the plasma for $CuSeO_4$ and ZnSe exhibited slightly elevated responses in Figure 2.3a compared to the organic species (and slightly lower responses in Figure 2.3b). Clearly, the nebulizer flow rate may cause a complete reversal in plasma robustness if not optimized correctly; it would seem that the higher nebulizer flow rate was overloading the plasma, causing a slight cooling effect within the central channel.

Although these percent changes in Mg intensity ratio do not seem very significant, they still exceed the fluctuation due to normal plasma processes, and this same trend was found to be reproducible, suggesting that a slight plasma temperature increase might be occurring for these particular species when run under optimized conditions. It is unlikely that the presence of Na is creating any kind of easily ionizable element effect, as the Na is present at too low a concentration to observe any such effect, although its presence could be affecting the way the droplets vaporize or atomize, changing the amount of energy required for these processes to occur, and so plasma temperature and robustness might be related to compound-dependent responses.

2.4.5 Droplet Properties

2.4.5.1 Addition of Surfactant

The results of the analysis of the Se compounds before and after the addition of 0.1% surfactant are shown in Table 2.3a and 2.3b, respectively. As expected, all species exhibited a slight increase in signal with the addition of Triton X-100, due to enhanced

nebulizer efficiency. Although the slopes of $(SeCys)_2$, Se^0, and Na_2Se (PB) did not experience any significant change with the addition of surfactant, SeMet increased from 89 to 96% of the slope of Se^{VI}, while SMSC increased from 94-104% with the addition. These increases were considered significant changes, although the reason as to why only those two species should exhibit any differences was unknown. Therefore, it was concluded that the surface characteristics of the droplets for each individual species might play a minor role in compound-dependency.

2.4.5.2 Droplet Size Distribution

Results of analyzing the calibration slopes of the Se standard solutions using the PerkinElmer experimental nebulizer, shown in Table 2.3c, revealed a more than 10-fold increase in signal, and although Na_2Se (PB) was still less sensitive than all of the other standards, it appeared that less difference was seen between Se^{VI}, SeMet, and SMSC. Dimethyldiselenide, too, which previously had been reporting approximately 91% of selenate's slope, was now 103%. Therefore, it was believed that droplet size distribution might offer a possible explanation for compound-dependent responses.

2.4.5.3 Aerosol Ionic Redistribution

The results of the multiple nebulization experiment, shown in Table 2.4, show a slight increase in sensitivity for the nebulized fraction collected in the waste beaker in comparison to the initial fraction that reaches the spectrometer. While the sensitivity of both Se^{VI} and Na_2Se (PB) species increased relative to their respective

initial readings, it seemed that the fraction of Se^{VI} that went to waste actually had a larger initial increase (7%) than the waste fraction of Na_2Se (PB, 2%), contrary to what was expected. Because these fractions were collected straight out of the spray chamber, it was assumed that little to no evaporation of solvent had occurred to produce an elevated concentration in the re-nebulized solutions. Therefore, it was concluded that aerosol ionic redistribution was likely not the cause of these compound-dependent responses.

2.4.6 Oxidation States of the Species

Results of the microwave digestion using both HNO_3 and H_2O_2 as oxidizing agents, shown in Table 2.5, revealed an almost reversal in compound-dependency trends, suggesting that complete oxidation to the +6 state was achieved by many of the species under investigation. Rather than all slopes being equal, though, with the exception of Na_2Se (PB), which still only produced a response equal to roughly half of the slope of Se^{VI}, many of the sensitivities were significantly greater than the slope of Se^{VI}. The most notable among these were ZnSe, Se^0, and $CuSeO_4$, which were 11, 13, and 18% higher, respectively. Selenate's slope, however, was found to be significantly different from all species with the exception of SeMet. Because the sensitivity of Na_2Se (PB) was still very low, it was speculated that complete oxidation had not occurred, although the difference in sensitivity is most likely not entirely a result of the -2 oxidation state of Se in Na_2Se, since ZnSe is also in the -2 state but produces a greater signal than Se^{VI} after oxidation.

It was concluded that a digestion procedure capable of breaking the Se bonds

and forcing it into solution can help lessen differences in compound-dependent sensitivities of some species, although it may make others worse, such as ZnSe, Se^0, and $CuSeO_4$. It was also suspected that the Na_2Se obtained from Pfaltz & Bauer was not what the manufacturer claimed it to be.

2.4.7 Colloidal Species

Results of the colloidal Se experiment, shown in Table 2.6, suggest that the synthesized colloidal solution produced a signal comparable to that of Se^{VI}, and so the idea that Na_2Se (PB) produced such low signals as a result of being a colloidal solution was dismissed. However, because the preparation step did not follow Stroyuk's[32] exactly, it is perhaps possible that a colloidal solution was not actually made. This bears further investigation.

2.5 Conclusions

Evidence of these compound-dependent responses in the literature, as well as new experimental evidence, was presented to offer further proof of their existence. During the course of this work, it was found that most Se species exhibited sensitivities that were approximately 5-10% lower than those of Se^{VI} and Se^{IV}. On the other hand, Na_2Se, purchased from two different vendors, gave widely different results (the material from Pfaltz & Bauer gave a response that was approximately 50% lower than selenate's slope, whereas the material from Sigma-Aldrich gave a response that was 5-10% higher). Based on experimental evidence, it was concluded that both "sodium selenide" materials, from Pfaltz & Bauer and Sigma-Aldrich, must

not be what the manufacturers claim; the consistently low signal, in the case of the compound from Pfaltz & Bauer, and the consistently high signal, in the case of the compound from Sigma-Aldrich, were not likely a result of compound-dependency, but rather a result of errors in the concentration of the solutions prepared arising from the mislabeling.

Although the reason for this phenomenon of compound-dependent response is still not clearly understood, it is believed that brands/models of instruments, sample introduction systems, and instrument/plasma operating conditions are not the main cause. It is also unlikely that the formation of oxides or refractory compounds, differences in volatility, or effects from carbon or easily ionized elements are the cause, although the results of experiment that investigated these phenomena suggest that the plasma becomes hotter when aspirating the inorganic species, producing a slightly elevated signal response. The reason for this change in temperature is not known, but is possible that it may be related to the physical properties of the droplets or the mechanism by which different species desolvate and/or atomize.

While minor differences of 5-10% in many of the responses may not seem so significant as to alter entire conclusions of past results, such differences do introduce error and raise questions about the validity of the results. Calibration using the exact species under study may be necessary for future studies in order to ensure greater accuracy is achieved. Strong oxidation of the compounds prior to analysis, addition of surfactant, or using a nebulizer capable of producing a very narrow distribution of small droplets sizes may also help to minimize the differences observed between species response factors.

It is hoped that the results described in this section will raise awareness of this compound-dependent response phenomenon and invite researchers to turn a critical eye towards past and future research.

2.6 Addendum

The differences between the two materials, each designated as "sodium selenide" from Pfaltz and Bauer, Inc. and Sigma-Aldrich Co., were astounding. Not only were the instrumental signal responses different for the two materials [Na_2Se (PB) gave a signal response roughly 50% less than expected, whereas Na_2Se (SA) yielded a response approximately 5-10% higher than expected], but the appearances of these two materials were also very different, *i.e.* Na_2Se (PB) was a clear/white, crystalline material, as expected, whereas Na_2Se (SA) resembled the appearance of Se^0, being a fine, dark-purple powder. Images of both materials under a microscope are shown in Figure 2.4. The Na_2Se (SA) was distinguishable from Se^0, however, because the "Na_2Se" was soluble in water, whereas Se^0 is not. The Na content of the Na_2Se (PB) material was also lower than expected: it was determined experimentally that the molar ratio of Na:Se, instead of being 2:1, was instead closer to 0.85:1.

X-ray diffraction (XRD) analysis using an X'Pert Pro X-ray diffractometer (PANalytical, Westborough, MA) was performed on the two Na_2Se compounds, in an effort to identify the compounds by their crystal structures. It was hypothesized that the white compound, Na_2Se (PB), might possess waters of hydration, which could explain why the instrumental signal response for Se was consistently low, as the solution concentration, therefore, would have been lower than that calculated for the

anhydrous salt. According to Sangster and Pelton,[35] Na_2Se can be hydrated with 4.5, 9, or 16 waters of hydration. While a few of the results, in retrospect, could be explained by the compound possessing 9 waters of hydration, more often than not, the calculated molecular weight of the compound is more consistent with 7 waters of hydration, which is not one of the allowed numbers. Furthermore, the XRD spectrum (see Figure 2.5[36]) for this compound fit neither the crystal structure of pure Na_2Se,[35] nor those of $Na_2Se{\cdot}9H_2O$,[37] Na_2Se_2,[38] or Na_2SeO_3,[39] although it is possible that the compound contains a mixture of several compounds or of several waters of hydration. On the other hand, the Na_2Se (SA), which was a dark-purple color, was hypothesized to be disodium diselenide (Na_2Se_2), a known purple compound that produces dark red solutions; if this were the case, it could explain the slightly elevated signal responses by ICP-OES and ICP-MS. It is worth mentioning that the label for the Na_2Se (SA) compound states, "Purity and identity have not been verified," and so, perhaps, it is possible that the wrong compound was packaged in that particular bottle (lot number AMS000983). The XRD results for this compound, shown in Figure 2.6,[36] however, did not match the crystal pattern of Na_2Se[35] nor Na_2Se_2,[38] and in fact, showed many similarities (although slightly shifted) to that of sodium selenite,[39] even though sodium selenite is white. Again, a mixture of sodium selenite and something else, such as elemental Se, might possibly explain the color, the solubility, and the XRD results. In summary, while the true identities of these two "Na_2Se" compounds are not known, it is certain that neither is the pure compound.

2.7 Acknowledgments

I would like to thank PerkinElmer for financial support and for the provision of the instrumentation. I would also like to thank Juan Ivaldi, for helpful discussion, Edward Voigtman, for assistance with statistical analysis of the calibration slopes, Elena Dodova, for laying the groundwork for this project, and Kevin Kittilstved, for help with the XRD measurements and interpretation of the data.

2.8 Tables and Figures

Table 2.1 Instrument conditions and other experimental parameters

Optima 4300 DV	
RF Power	1500 W
Nebulizer gas flow	0.55 L min^{-1}
Plasma gas flow	15 L min^{-1}
Auxiliary gas flow	0.2 L min^{-1}
Pump rate	1.0 mL min^{-1} (0.045 in. i.d.)
Online addition pump rate	0.1 mL min^{-1} (0.015 in. i.d.)
Nebulizer	GemCone
Spray chamber	Glass Cyclonic
Wavelengths monitored	Se 196.026, Mg 280.271, Mg 285.213 nm
Optima 8300 Flat Plate	
RF Power	1500 W
Nebulizer gas flow	0.55 L min^{-1}
Plasma gas flow	15 L min^{-1}
Auxiliary gas flow	0.2 L min^{-1}
Pump rate	2.0 mL min^{-1} (red-red)
Nebulizer	PerkinElmer, Inc. experimental nebulizer prototype #9192
Aerosol delivery system	ADS #3, fluid feed #G-5
Nebulizer frequency	77.5 kHz
Nebulizer duty cycle	8%
Nebulizer burst period	1000 Hz
Wavelengths monitored	Se 196.026 nm
Elan 6100	
RF Power	1500 W
Nebulizer gas flow	1.01 L min^{-1}
Nebulizer	GemTip Cross-Flow II
Spray chamber	Scott
Detector mode	Dual mode
Sampler/skimmer cones	Nickel
Scanning mode	Peak hopping
Dwell time	100 ms per point
Number of sweeps/read	10
Number of reads/replicate	1
Number of replicates	10
Isotopes monitored	^{78}Se
MARSXpress microwave system	
Vessels	Xpress vessels, 75 mL Teflon
Power	1600 W
Percent power operation	100%
Ramp time	20 min
Maximum temperature	180 ºC
Hold time	40 min
Cool down time	60 min

Table 2.2 Typical instrument responses for Se species using ICP-OES and ICP-MS

	a: ICP-OES (Optima 4300 DV)				b: ICP-MS (Elan 6100)			
Species	**conc. ($\mu g\ g^{-1}$)**	**avg. intensity**	**slope (cps/ $\mu g\ g^{-1}$)**	**% of slope, relative to that of selenate**	**conc. ($\mu g\ g^{-1}$)**	**avg. intensity**	**slope (cps/ $\mu g\ g^{-1}$)**	**% of slope, relative to that of selenate**
Selenate	9.97	11379	1146	100	10.1	11599	639	100
Selenite	9.73	10928	1127	98	10.2	11598	633	99
Selenious acid	10	11139	1115	97	10.5	11738	630	99
SeMet	10.1	10704	1069	93	11.2	11943	609	95
SMSC	10.1	10795	1072	94	10.9	11867	617	97
$(SeCys)_2$	9.96	10974	1106	97	10.9	11914	623	97
Na_2Se (PB)	10.4	5643	545	48	10.8	8643	325	51
Na_2Se (SA)	10.6	13051	1236	108	9.84	11764	673	105
Se^0	10.1	11077	1104	96	10.7	11957	639	100
ZnSe	9.67	10671	1107	97	10.4	11635	626	98
$CuSeO_4$	10	11134	1114	97	10.6	11930	638	100
DMDSe	9.98	10316	1038	91	9.44	10873	607	95

Table 2.3 Calibration data for several Se species under different conditions using ICP-OES.[a]

a: solutions without surfactant

Species	conc. ($\mu g\ g^{-1}$)	avg. intensity (cps)	slope (cps/ $\mu g\ g^{-1}$)	%
Selenate	10.1	10541	1049	100
SeMet	10.2	9491	936	89
SMSC	10	9854	986	94
$SeCys_2$	9.95	9940	1001	95
Se^0	9.91	9922	1003	96
Na_2Se (PB)	9.92	5030	509	48

b: solutions with 0.1% Triton X-100

Species	conc. ($\mu g\ g^{-1}$)	avg. intensity (cps)	slope (cps/ $\mu g\ g^{-1}$)	%
Selenate	10.1	11875	1179	100
SeMet	10.2	11522	1133	96
SMSC	10	12283	1225	104
$SeCys_2$	9.95	11378	1142	97
Se^0	9.91	11365	1145	97
Na_2Se (PB)	9.92	5519	555	47

c: solutions run using experimental nebulizer

Species	conc. ($\mu g\ g^{-1}$)	avg. intensity (cps)	slope (cps/ $\mu g\ g^{-1}$)	%
Selenate	10.5	178517	17049	100
Selenious acid	9.89	178247	18032	106
SeMet	9.72	163459	16825	99
SMSC	9.96	166945	16762	98
Na_2Se (PB)	9.97	87933	8821	52
DMDSe	10.1	177263	17555	103

[a] Percentages refer to the slope of the species, relative to the slope of selenate during that experiment.

Table 2.4 Se^{VI} and Na_2Se (PB) after multiple nebulizations.

Species	avg. intensity (cps)	% of slope, relative to first nebulization
Se^{VI} - first	12308	100
Se^{VI} - second	13129	107
Se^{VI} - third	13409	109
Na_2Se (PB) - first	6703	100
Na_2Se (PB) - second	6836	102
Na_2Se (PB) - third	6846	102

Table 2.5 Comparison of Se species' calibrations after microwave digestion.

Species	conc. (µg g^{-1})	avg. intensity (cps)	slope (cps/ µg g^{-1})	% of slope, relative to selenate
Selenate	10.4	9657	931	100
Selenite	10.0	9597	961	103
Selenious acid	10.0	9764	982	105
SeMet	9.72	9023	929	100
SMSC	10.0	9479	949	102
$SeCys_2$	9.87	9657	980	105
Se^0	10.1	10631	1051	113
Na_2Se (PB)	10.0	4798	480	52
ZnSe	9.92	10272	1037	111
$CuSeO_4$	9.77	10704	1097	118

Table 2.6 Comparison of Se^{VI}, Na_2Se (PB), and colloidal Se calibrations.

Species	conc. (µg g^{-1})	avg. intensity (cps)	slope (cps/ µg g^{-1})	% of slope, relative to selenate
Selenate	10.2	25781	2524	100
Colloidal Se	10.1	25086	2476	98
Na_2Se (PB)	10.4	12411	1195	47

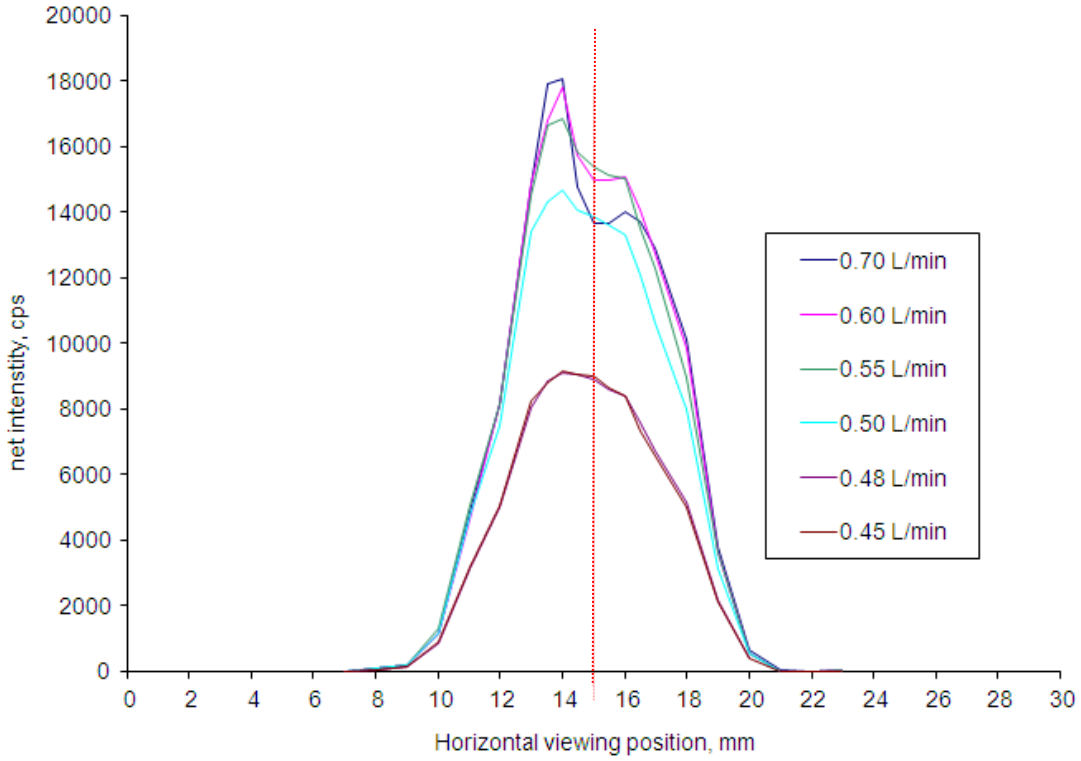

Figure 2.1 Intensity profile for Se^{VI} as a function of axial viewing position and nebulizer gas flow. Identical profiles, varying only in the magnitude of the y-axis, were observed for SeMet and Na_2Se (PB). The center line drawn at 15 mm indicates where maximum intensity is observed for Mn.

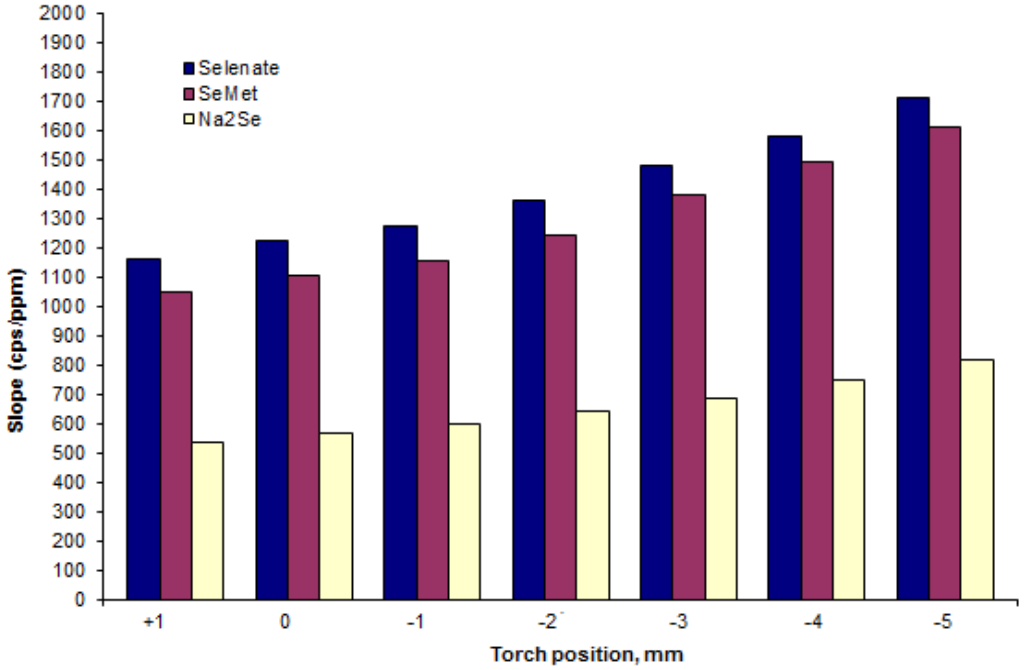

Figure 2.2 Calibration slopes as a function of the distance between injector tip and load coil. The slopes of Se^{VI}, SeMet, and Na_2Se (PB) are shown.

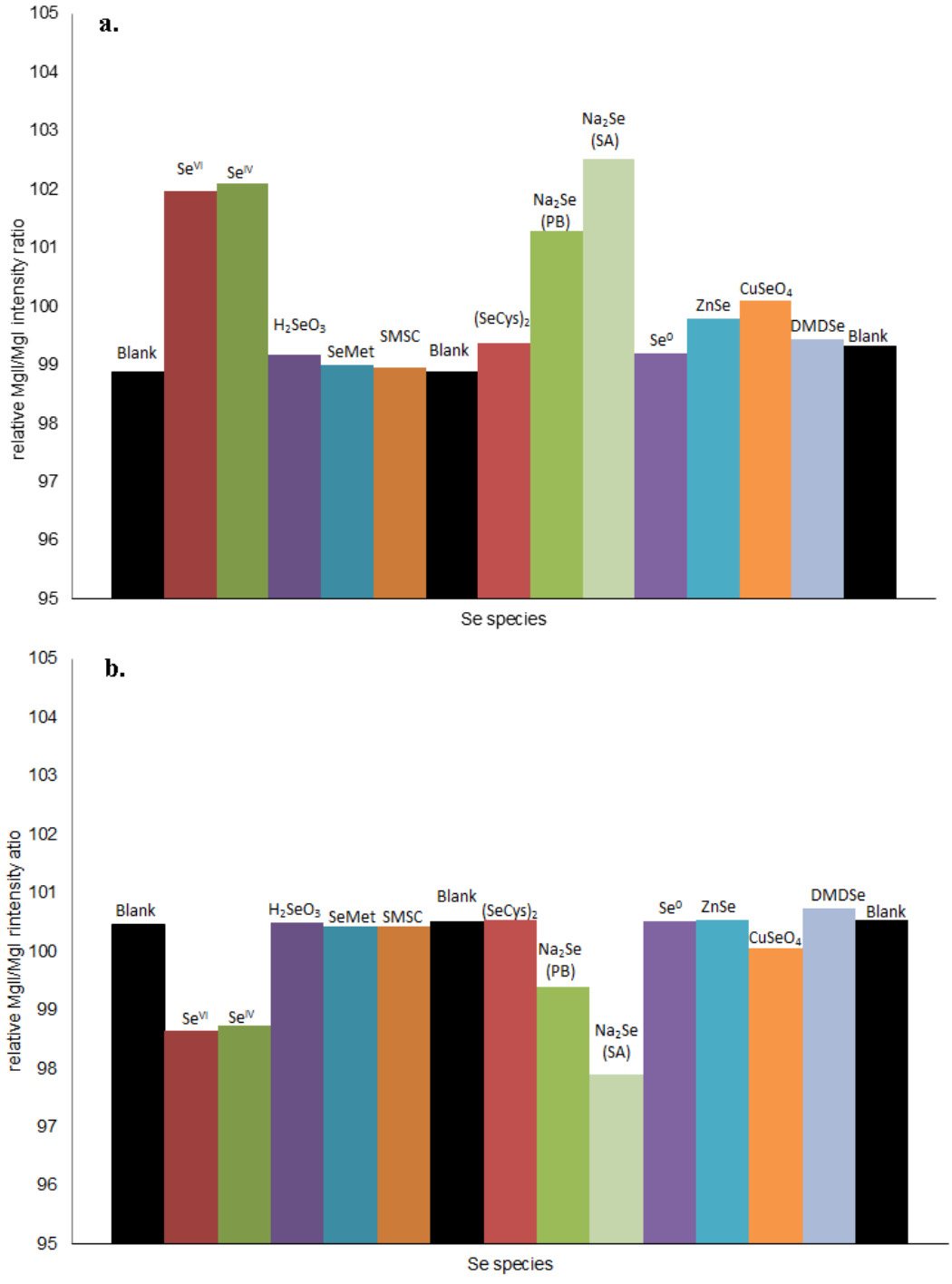

Figure 2.3 Mg intensity ratios versus Se species. The individual Se species were run at an optimized nebulizer flow rate of 0.55 L min-1 (a) and a non-optimized flow rate of 0.70 L min-1 (b).

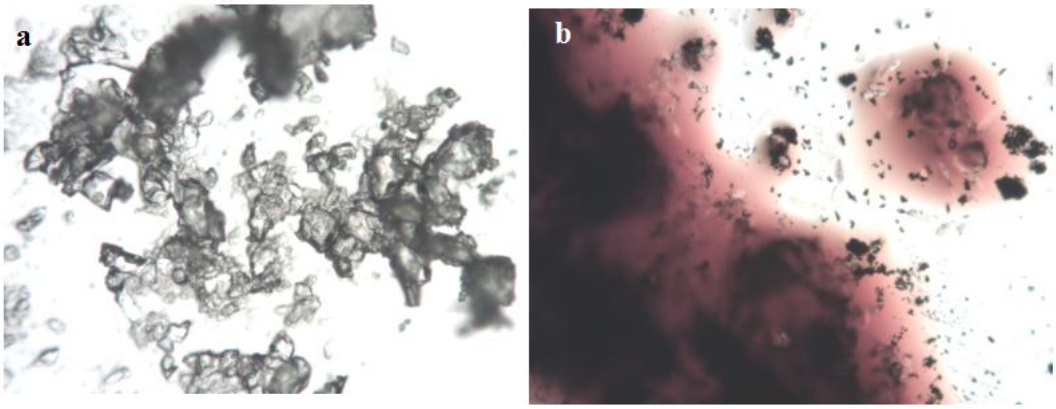

Figure 2.4 Microscope images (10x optical zoom) of (a) Na_2Se (PB) and (b) Na_2Se (SA).

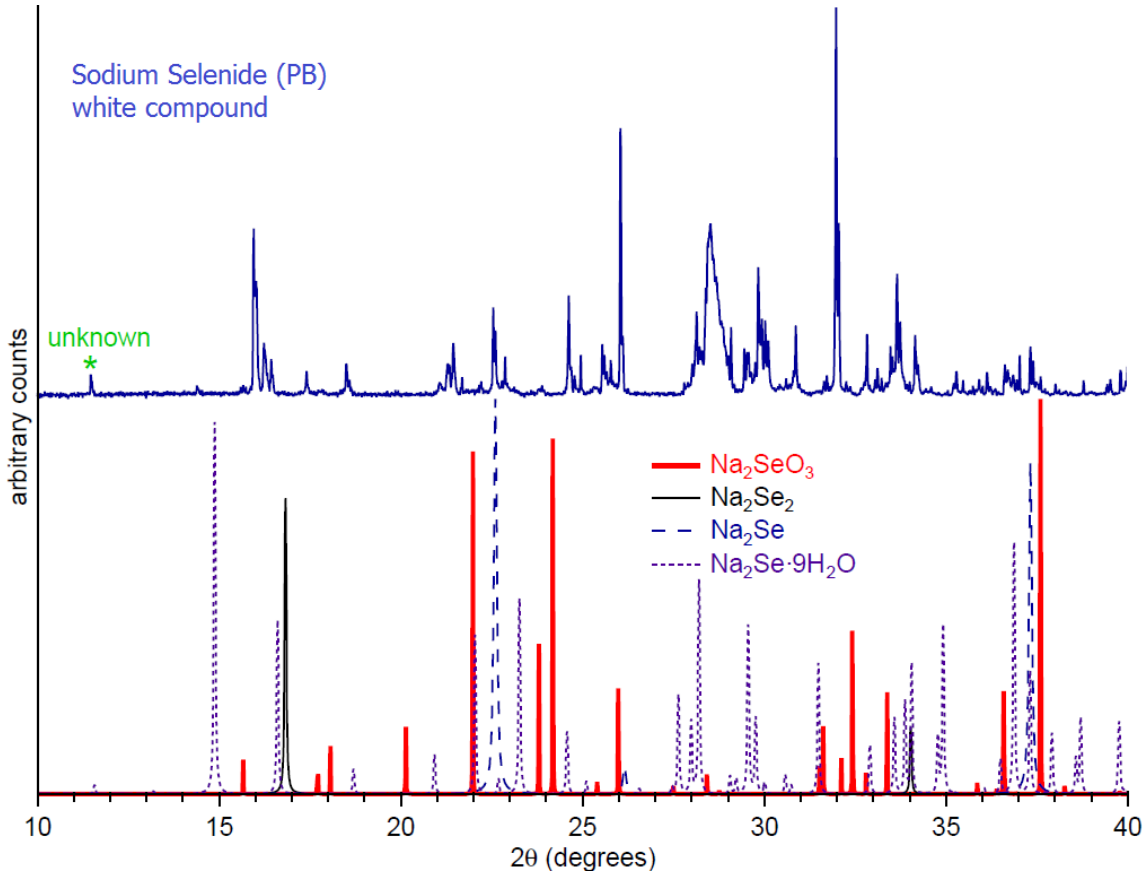

Figure 2.5 X-ray diffraction pattern of Na_2Se (PB), compared to the patterns of four other selenium compounds.[35-39]

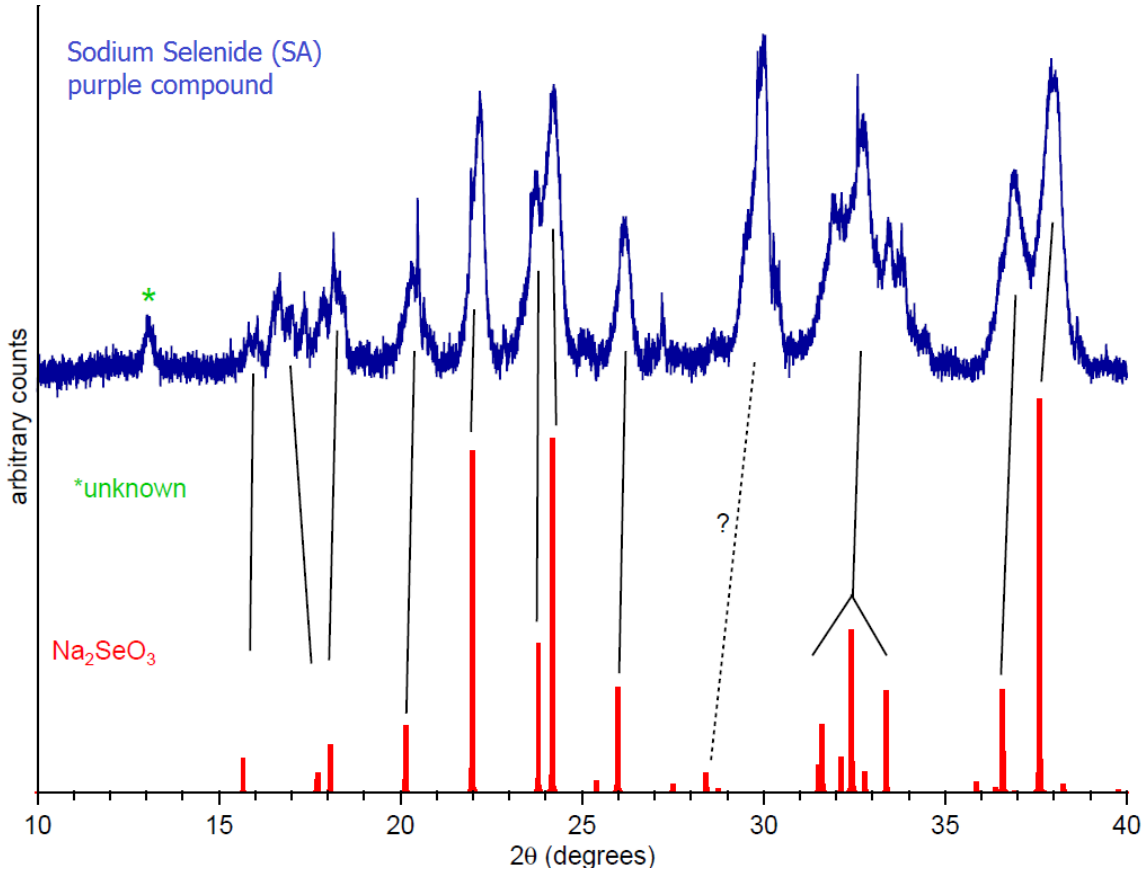

Figure 2.6 X-ray diffraction pattern of Na_2Se (SA), compared to the pattern of Na_2SeO_3.[36,39]

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CHAPTER 3

SIMULTANEOUS DETERMINATION OF INORGANIC AND ORGANIC SELENIUM SPECIES IN DIETARY SUPPLEMENTS USING ANION-EXCHANGE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

3.1 Introduction to Selenium

Research into the analytical and nutritional chemistry of selenium (Se) and its compounds, and their separation and speciation, has been reviewed in 2008 by Amoako.[1] Selenium is an essential nutrient and can be found in soil, plants, and animals.[2] In the environment, it exists predominantly as elemental selenium (oxidation state 0), selenite (oxidation state +IV), selenate (oxidation state +VI), selenide (oxidation state –II), selenoamino acids, and a wide variety of other organic forms.[3] A listing of a number of common Se compounds and their structures is provided in the Appendix. Geographic region largely determines the amount of selenium present in soil; for example, certain regions of the United States, Venezuela, and the former USSR contain high amounts of selenium (over 1000 $\mu g\ g^{-1}$), whereas other areas such as parts of Europe and China, Korea, and New Zealand are very deficient (less than 0.1 $\mu g\ g^{-1}$).[4] Plants grown in selenium-rich soil can readily incorporate the selenium compounds into their shoots and roots, thus introducing selenium into the food chain, although complexation with metals, such as iron and aluminum in an acidic environment can decrease the extent of uptake.[5] Selenium is taken into the human body mainly through diet;[5] the primary source comes from wheat[6] in the United States, although selenium is also found in some vegetables, and approximately 20% of the total ingested selenium comes from meat products.[5] It has been shown by Irons et al.[7] that selenoproteins can demonstrate anti-cancer properties in

mice, and, according to Rayman et al., "It seems likely that antioxidant selenoproteins may be of benefit in counteracting diseases of oxidative stress."[8] Furthermore, Pyrzynska reports that, "selenium compounds also catalyze the reactions of intermediate metabolism and inhibit the toxic effects of heavy metals such as arsenic, cadmium and mercury."[9]

Selenium deficiency in humans and animals can lead to a number of metabolic diseases, which can impair growth and reproduction; humans deficient in selenium are susceptible to developing Keshan disease, which causes cardiomyopathy, Kashin-Beck disease, which is a form of arthritis that can lead to deformity, and cretinism,[5] which is a form of hypothyroidism leading to stunted physical and/or mental growth.[10] On the other hand, selenium poisoning, which is a condition known as selenosis, can lead to respiratory, gastrointestinal, and cardiovascular problems.[5] Skin and tooth discoloration, hair loss, tooth decay, nail deformation, garlic-breath, and physical weakness are often associated with long-term exposure to high levels of selenium.[5]

3.1.1 Chemical Form and Supplementation

According to Ganther, "It is important to keep in mind that the biological activity of selenium is an expression of selenium in a wide variety of chemical compounds, and not the element, per se".[11] Therefore, it is important to emphasize that, when discussing selenium as it occurs in foods, plants, and other matrices, typically a selenium-containing compound is being referred to, not selenium in its elemental form. Although selenium is an essential trace element for human health, the form in which it occurs can have either beneficial or toxic effects;[8] furthermore, though essential, there is an upper limit for selenium intake, although there exists much ambiguity in the literature regarding dosage.

For example, there needs to be a unified system of reporting dosage, as "RDA" can be defined as either "recommended daily allowance" or "recommended dietary allowance". Also, some literature reports dosage in terms of μg day^{-1}, whereas others report it as mg kg^{-1} of body weight, or even mg kg^{-1} of the diet. The recommended dietary allowance for selenium, according to the National Research Council, is 55 μg day^{-1} for both men and women,[12] with 400 μg day^{-1} being the upper limit;[13] adverse effects are generally not realized until double this amount, but the 400 μg day^{-1} allows for a margin of safety, given that the bioavailability and toxicity of Se depends largely on its chemical form.[14] Because it has been demonstrated that certain forms of selenium may have anti-cancer properties,[15] many researchers have sought to find out which, if any, particular forms of selenium are the ones needed to assuage certain ailments. However, according to Amoako et al.,[16] "The marketing of selenium supplements in recent years has increased but regulation with respect to quality, storage conditions, stability and content of selenium supplements is problematic. Further selenium speciation for selenized yeast and selenium supplements is needed to quantify active components and potential anti-carcinogenic components."

3.1.2 Selenium and Cancer Chemoprevention

3.1.2.1 Nutritional Prevention of Cancer Trial

For a number of years, it has been conjectured that selenium may have anti-carcinogenic properties. Through their studies, Ip and Ganther[17] concluded that, although the exact mechanism still is not known, "some active species of selenium with anti-tumorigenic potential is formed by the metabolism of selenium and is generated only

when the supply of selenium is maintained above a certain minimal level." Health problems, such as cancer, cardiovascular disease, and neurodegenerative diseases, are typically linked to oxidative stress, an overproduction of reactive oxygen species (ROS) within the body that can damage lipids, proteins, and DNA; on the other hand, some ROS are necessary for healthy immune response, as they protect against infection.[18] A recent, comprehensive review by Ramoutar and Brumaghim[18] discusses how studies suggest that, under certain conditions, some selenocompounds exhibit both antioxidant (i.e. protecting cells against harmful ROS) and pro-oxidant (i.e. producing ROS to combat cancerous or other harmful cells) properties. The precise mechanism for these effects, however, is still unknown, although several theories exist.[18]

In 1996, Clark et al.[15] demonstrated in the Nutritional Prevention of Cancer (NPC) Trial, which began in 1983 and lasted over seven years, that the incidence of certain cancers was reduced in participants taking daily supplements of selenium, although there was no statistical evidence supporting that supplementation reduced the incidence of the trial's primary target, skin cancer.

According to Thorpe et al.,[19] "the ideal chemopreventive agent should be non-toxic, cheap and with good bioavailability following oral administration." Participants in the NPC Trial were given 200 μg day^{-1} of selenium in the form of selenium-enriched baker's yeast (*Saccharomyces cerevisiae*), also called "high-selenium baker's yeast", or simply "selenized yeast" that is believed to contain mostly organic forms of selenium, approximately 60% of which is in the form of selenomethionine.[20] However, it is of note that the other constituents of the yeast are, as of yet, still not fully identified, nor are the potential anti-carcinogenic forms known.[16,20] According to Dumont et al.,[21] "most often,

the suppliers provide information on the total concentration of Se, but information on the species present is scarce or even absent." Furthermore, not all selenium-enriched baker's yeast is the same, as it varies in composition from sample to sample,[5] thus compromising the credibility of the trial. According to Rayman et al.,[8] "Se-yeast . . . is not a defined form of Se. There is considerable variability in products described as Se-yeast which is reflected in the species composition." They then go on to describe how selenium can organically bind to yeast when the yeast is fermented in the presence of selenium, thus producing "selenized yeast"; however, Rayman et al.[8] point out a major problem with this process, one which can be misleading to consumers: "With reputable manufacturers, the percentage of Se that is organically bound should be greater than 90% and more than 80% should be bound to yeast proteins, including cell-wall proteins. However, in some products, the percentage of sodium selenite is such that most of the Se is clearly not bound to the yeast; at worst, there may merely be a mixture of sodium selenite and yeast, the Se not being bound to the yeast . . . While they may be capable of increasing the production of selenoproteins, they will be less good at increasing plasma Se."

Additionally, analytical studies have shown that "selenized yeast" changes in selenium speciation on storage at ambient and/or elevated temperatures. Specifically, selenomethionine is oxidized to selenomethionine selenoxide continually after supplement formulation, and the metabolite *S*-(methylseleno)cysteine is formed in proportionally increasing amounts with time, while selenomethionine content decreases proportionally.[16] No clinical or nutritional studies on these metabolites have been performed.

The original NPC Trial's primary focus was to monitor the risk of recurrent non-melanoma skin cancer among participants, with the incidence of prostate cancer, colorectal cancer, and cancer mortality being a secondary focus. Following the publication of the NPC's results, many subsequent investigators analyzed the data retrospectively for these other cancers. For example, Reid et al. re-evaluated the NPC Trial's results to determine the effect of selenium supplementation on lung cancer,[20] prostate cancer,[22] and colorectal cancer.[23] The results of each of these studies suggested that the risk of cancer was diminished with selenium supplementation, so long as the participant was in the lower tertile range of baseline plasma selenium.[20,22,23] These tertiles were typically defined as lower (approximately $\leq$ 106 ng mL^{-1} baseline plasma Se concentration), middle (approximately 106-123 ng mL^{-1}), and upper (approximately $\geq$ 123 ng mL^{-1}).[22] Participants with histories of smoking also appeared to benefit more so than nonsmokers, having higher reductions in the risk of these cancers.[20,22,23]

Despite the agreement among these reports that selenium supplementation may decrease the risk of certain cancers, provided the individual has low baseline plasma selenium at the onset of supplementation, the authenticity of these results may need to be questioned; these data were all analyzed retrospectively, while some needed to correct for bias. For example, the re-analysis of the effect of supplementation on prostate cancer was subject to bias; 35% of the placebo group of men with abnormal amounts ($\geq$ 4 ng mL^{-1}) of prostate-specific antigen (PSA) underwent biopsies during the study period, whereas only 14% of the supplemented group of men with abnormal PSA levels were biopsied, thus leading to bias within the supplemented group for the detection of prostate cancer.[22] Furthermore, the validity of some of these biomarkers, such as PSA, that serve

as surrogate measurements, need to be evaluated for their ability to predict disease reliably.[19] Also, not all variables were taken into account in these studies; for example, age, gender, baseline plasma selenium levels, and smoking habits were recorded, but other variables, such as diet, physical activity, and other potential risk factors, were overlooked according to Duffield-Lillico et al.[22] Lastly, not only did all members of the NPC Trial exhibit non-melanoma skin cancer, but also a large number of them were former or current heavy tobacco users.[20] Duffield-Lillico et al. conclude that a trial involving participants with no history of cancer of any kind and an equal amount of smokers versus nonsmokers would be much more beneficial in understanding the effects of selenium supplementation, as it is currently unclear as to how these results may vary when supplementation is applied to cancer-free, non-smokers.[20] Stranges et al.[24] also agree that the findings of the NPC Trial cannot be generalized to other groups of people, as the mean age of the trial's participants was 63.2 years and all individuals were from low-selenium areas of the United States.

Another retrospective analysis of the data from the NPC Trial had slightly different results; Stranges et al.[24] looked at the incidence of type II diabetes on the participants in the NPC Trial and found that, rather than observing a decrease in incidence within participants of the lower tertial range, they instead found no significant effect on those participants, whereas participants in the upper baseline plasma selenium range observed an *increased* risk for type II diabetes. Stranges et al. offer, as a possible explanation for this observation, that participants who already had higher, natural levels of selenium in their bodies may have experienced an overdose with the supplementation, leading to over-expression of the enzyme glutathione peroxidase. Glutathione

peroxidase, which contains four selenocysteine residues, is the most abundant selenoprotein in mammals,[24] and it plays an important role in protecting membranes from damage by peroxides.[9] Over-expression of this enzyme has been known to lead to insulin resistance, and consequently, type II diabetes.[25]

3.1.2.2 Selenium and Vitamin E Cancer Prevention Trial

To remove some of the issues of bias and retrospective analysis in regard to the NPC Trial, another trial was initiated in 2001 called SELECT (Selenium and Vitamin E Cancer Prevention Trial). This was designed to run for seven to twelve years, and aimed to observe the effect of selenium (as *L*-selenomethionine, 200 μg day^{-1}) and Vitamin E (as *all* rac-α-tocepheryl acetate, 400 international units (IU) day^{-1}) supplementation on 35,000 participants, with the incidence of prostate cancer as its primary focus and lung, colorectal, and overall cancer as its secondary targets.[26] These men were relatively healthy, having low levels of PSA ($\leq$ 4 ng mL^{-1}) and no known cases of pre-existing prostate cancer.[27] Furthermore, the participants in SELECT had higher initial plasma Se levels (135 ng mL^{-1}), relative to the participants of the NPC Trial (113 ng mL^{-1}).[28] According to Lippman et al.,[26] the decision to use selenomethionine arose from a unanimous vote of selenium experts, who met to discuss dosage and chemical form in 1998. According to Uden,[29] there still remained debate as to the actual form of the selenium supplement to be employed; one argument was that a supplement of 'selenized yeast' with manufacture method and properties as similar as possible to that used in the NPC trial should be used to replicate that study most closely. However, as noted above, analytical chemical studies had indicated that formulated selenized yeast was subject to

degradation and production of organoselenium species other than selenomethionine and thus the speciation of selenium in dosage form was subject to variation. In 2001 it was confirmed that the doubts as to uniformity and stability of selenized yeast militated against its use in the SELECT program, and a better course of action was to restrict the active supplement to *L*-selenomethionine. It has been shown that *L*-selenomethionine can be metabolized by the human body, but the *D*-enantiomer cannot, and is instead excreted unchanged.[30]

In retrospect this decision to use *L*-selenomethionine, although made responsibly, clearly altered the dosage form from that of the NPC trial and may indeed have resulted in the outcome described below.[29] The proposal, strongly held by some, that the SELECT study should also include comparative parallel results from both selenized yeast and selenomethionine supplementation, could not be implemented because of lack of funding at the Nutritional Cancer Institute (NCI).[29] Also, although, at the time, new evidence suggested that monomethylated forms of selenium may have better anti-carcinogenic properties, the panel still agreed upon selenomethionine, given that these relatively newer compounds had yet to be fully tested for safety and were not commercially available.[29] More recently, it has been confirmed that the methylated metabolites indeed have greater anti-cancer properties than non-methylated forms.[31]

The SELECT study showed conflicting results compared to those of the NPC Trial, bringing the study to a halt after only seven years. A meeting held by the SELECT Data and Safety Monitoring Committee (DSMC) in September of 2008 regarding the supplementation of those 35,000 participants declared that selenium and vitamin E did not prevent prostate cancer within that population of test subjects; in fact, according to

the SELECT website, "The data to date suggest, but do not prove, that vitamin E may slightly increase the chance of getting prostate cancer, and that selenium may increase the chance of developing diabetes mellitus."[32] A possible explanation for these contradicting results, offered by SELECT, is that the NPC study headed by Clark et al. did not focus solely on prostate cancer, and so therefore, the participants were not regularly screened for it, whereas prostate cancer was the primary focus of the SELECT trial, and was therefore monitored more closely. However, according to Lippman et al.,[26] as a result of budget constraints, the SELECT trial was not capable of "collecting additional data, e.g., clinical information such as other medications, and biospecimens for research." Other factors, too, such as high estrogen levels, low testosterone levels, and diets rich in fatty acids, are known to increase the risk of prostate cancer,[33] but were not monitored in SELECT. Furthermore, not only was it not mandatory for all participants to receive annual prostate cancer screenings or digital rectal exams (DRE), thus relying on participants' self-reports, but some of the participants had also been involved in a former prostate cancer trial, called the Prostate Cancer Prevention Trial (PCPT), in which participants were given the drug finasteride.[26] Keeping track of participant compliance may have been an issue, leading to potential bias.

Spallholz of Texas Technical University offers the bioavailability of the chemical species administered as another explanation for the results of the SELECT trial. Spallholz[34] states, "The recent apparent failure of the SELECT Trial is that dietary selenomethionine is lost to non-specific protein synthesis, is not "targeted" and does not redox cycle unless enzymes that generate methylselenol (methioninases) are sufficiently present in the cancer cells. A much better selenoamino acid for "chemoprevention" as

determined by animal studies . . . is *Se*-(methylseleno)cysteine . . . It too is not "targeted" at any specific cancer cell but it is not lost to protein syntheses thereby making the oral supplementation…of selenium effectively higher than an equivalent amount of selenomethionine." This theory follows that of Ip and Ganther,[18] who, in 1992, concluded that certain compounds, such as selenobetaine and *Se*-(methylseleno)cysteine demonstrate better anti-carcinogenic activity, in contrast to selenite and selenomethionine, the reason being that they produce monomethylated metabolites with more efficiency. Monomethylated metabolites may be the key behind cancer prevention, as Ip and Ganther[18] believe that compounds with higher degrees of methylation are more likely to be excreted. Furthermore, Ip and Ganther[18] state that selenite demonstrates better potency than selenomethionine and selenocystine, because the latter two compounds can be nonspecifically incorporated into proteins, thus lowering the anti-carcinogenic potential.

Another explanation, offered by Rayman,[35] is that risk reduction in certain cancers is closely linked to optimization of specific selenoproteins. According to Rayman,[35] approximately one-third of the participants of the NPC Trial were deficient in these selenoproteins, thence the results of the trial showed that supplementation reduced the risk of certain cancers; conversely, participants of SELECT already had sufficient levels of selenoproteins at the onset of the trial, thus showing little reduction of risk upon supplementation with selenium. In agreement, Taylor states that, "Micronutrient supplementation in an already well-nourished population, particularly at higher doses, is not useful, at least not in the later years of life."[36] Indeed, supplementing people who

already have optimal levels of selenium may lead to greater problems, just as the increased risk of type II diabetes among SELECT participants suggests.

It is extremely important to find out how selenium supplementation can affect humans, just as it is also important to identify the precise amounts and species that affect us beneficially or adversely. Selenium supplementation regimes involving between 30 and 200 μg dose^{-1} are available to the United States and Western Europe,[24] and so it is imperative that more work is performed in this field to ensure that supplementation is done with full knowledge of its effects.

3.1.3 Speciation

"The goal of analytical separation," according to Uden,[37] "is to achieve the greatest possible resolution and selectivity for the components of a complex sample, and thereby to obtain qualitative and quantitative information in an accessible and timely way." In many cases, the greatest separation and detection can be achieved through the use of hyphenated techniques. Uden states that "the goal of a hyphenated system is to enable more analyte characterization than mere monitoring of eluates. Molecular and elemental characterization are usual goals in speciation; the latter is a powerful basis for discerning qualitative and quantitative aspects of selenium chemistry."[37] According to Pyrzynska,[9] procedures used in speciation typically distinguish between two classes of selenium species: nonvolatile species (selenate, selenite, elemental selenium, and matrix-bound organic selenium compounds) and volatile organic species (volatile selenides).

3.1.3.1 High Performance Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry (HPLC-ICP-MS)

Separation, quantitation, and characterization continue to be the biggest problems associated with selenium speciation; the vast majority of recent research has been accomplished through the use of techniques that couple the separation capability of chromatography to the sensitive detecting ability of inductively coupled plasma mass spectrometry (ICP-MS).[5] For instance, high performance liquid chromatography coupled to ICP-MS (HPLC-ICP-MS), along with molecular MS, has been used in the last fifteen years to identify sixteen selenium compounds in urine.[38] Liquid chromatography, particularly high performance liquid chromatography, is well suited for speciation analysis, as it requires little sample preparation of liquid samples, can be performed at ambient temperatures, can achieve maximum separation capability by varying both stationary and mobile phase compositions, can achieve low detection limits, and is relatively fast.[9] Although ICP-MS is susceptible to isobaric interferences, it is less so when used primarily as the detector; further, interferences can be reduced by employing a dynamic reaction/collision cell.[37]

A wide variety of separation modes can be applied to selenium speciation with liquid chromatography. For instance, reversed-phase liquid chromatography can be used to separate a broad range of anions, cations, and neutral species,[5] as well as selenoamino acids.[39] Inorganic species can be separated via ion-exchange,[40] selenoproteins[41] and selenosugars[42] can be separated by size exclusion, and cationic selenium species can be separated by cation-exchange chromatography.[43] Despite the many different techniques available, however, few are efficient at separating both organic and inorganic species

simultaneously. More commonly, cation-exchange is used for the separation of the Se amino acids, whereas the separation of selenate from selenite is reserved for anion-exchange chromatography.[44]

There have been a number of recent studies using HPLC-ICP-MS for selenium speciation. For instance, Pan et al.[45] demonstrated complete separation of six selenium species (selenite, selenocystine, trimethylselenonium ion, selenoethionine, selenomethionine, and selenourea) in urine in less than ten minutes using reverse-phase ion-pair chromatography with ICP-MS detection; detection was possible even at concentrations as low as 0.1 μg L^{-1}. Unfortunately, the method was not well suited to speciate selenate, as it eluted after 40 min, although this was not an issue at the time as selenate is not typically a urinary metabolite. Similarly, Cuderman et al.[46] separated five Se species [selenite, selenate, selenocystine, selenomethionine, and seleno(methyl)selenocysteine] by anion-exchange HPLC-ICP-MS, but also needed to use a cation-exchange column because the selenocystine eluted in the void volume. Juresa et al.[43] monitored selenium metabolites quantitatively in rats, finding that the major species in background urine were "selenosugar 1" (methyl-2-acetamido-2-deoxy-1-seleno-β-D-galactopyranoside, 67%) and trimethylselenonium ion (13%). Kahakachchi et al.[47] identified *S*-(methylseleno)cysteine for the first time in the roots and shoots of *Brassica juncea* (Indian mustard) when grown in the presence of selenite or selenate. Amoako et al.[16] demonstrated that commercially-available supplements, including some of those used in the human intervention trials, vary in speciation depending on time and storage conditions, particularly selenomethionine, selenomethionine selenoxide, and *S*-(methylseleno)cysteine. Cankur et al.[48] showed using HPLC-ICP-MS that dill (*Anethum*

graveolens L.), grown in sodium selenite, has the highest relative concentration of *Se*-(methylseleno)methionine in the root, suggesting that the roots may be involved in selenium volatilization.

As pointed out in 2004 by Francesconi and Pannier,[38] there has been some confusion in the literature regarding selenium metabolites in urine matrices; incorrect names and/or structures have been applied loosely, especially for the selenosugars, and there has also been a great deal of contradiction as to which selenium species are the primary urine metabolites. For example, new evidence has emerged, suggesting that selenosugars are the major metabolites in urine, thus challenging the popular belief that the major constituent was trimethylselenonium ion.[38] Furthermore, they point out that very little has changed in the field of selenium speciation and quantification, even after Robberecht and Deelstra[49] in 1984 urged researchers to increase sample size and improve the care with which they run their experiments; Robberecht and Deelstra stated that the majority of the research surrounding this topic was contradictory and lacking proper data interpretation. Indeed, Francesconi and Pannier[38] state, "Much of the apparent variability and diversity of selenium metabolites appearing in the recent literature has stemmed from poor application of HPLC/ICPMS."

A possible reason for this apparent lack of accuracy is that, until recently, there were no reference materials for selenocompounds; high-selenium baker's yeast was often used as a standard, but, as previously noted, it is poorly-characterized and varies in composition from sample to sample.[5] However, recent international studies demonstrated that accurate results for total selenium and selenomethionine in yeast and wheat flour samples can be achieved, thereby providing reference materials for method validation

studies. These include the materials SELM-1, CCQM-P86 selenised yeast, and CCQM-K60 selenised wheat flour. Through a variety of techniques, it was agreed upon that the pharmaceutical yeast material SELM-1 contains 337.6 mg kg^{-1} (with standard deviation of 9.7 mg kg^{-1}, where n = 13) of total selenium, and 561.5 mg kg^{-1} (with standard deviation of 44.3 mg kg^{-1}, where n = 11) of selenomethionine, whereas an average of 95% (n = 9) recovery of selenomethionine from SELM-1 was achieved.[50] On the other hand, the CCQM-P86 yeast and CCQM-K60 wheat flour contain 337.6 ± 9.7 and 17.3 ± 0.07 mg kg^{-1} total Se, and 561.5 ± 44.3 and 28.3 ± 0.5 mg kg^{-1} SeMet, respectively, although they have not been officially certified, as of yet.[50,51]

3.1.3.2 Gas Chromatography (GC)

Gas chromatography (GC) is a technique commonly used to separate volatile selenium species, such as methylselenol, dimethylselenide (DMSe) and dimethyldiselenide (DMDSe). These species can be liberated from the sample matrix by heating, trapping on an adsorbent, and then releasing via thermal desorption before GC analysis.[9] Either packed or capillary columns can be used to achieve separation; packed columns have an advantage over capillary columns in that they have a much larger sample capacity, but conversely, the capillary columns are able to achieve much higher resolution.[9] Coupling an atomic emission detector (AED), mass spectrometer, or ICP-mass spectrometer, can further increase GC utility; for instance, GC-AED has been used to identify and quantify many volatile selenium species present in, or produced by, plants,[35] whereas Kremer et al.[52] have shown that GC-ICP-MS can monitor the amount of dimethylselenide in human breath periodically after the ingestion of selenite. Common

derivatizing agents for GC-AED, particularly for the determination of selenoamino acids such as selenocysteine, selenomethionine, and *Se*-(methylseleno)cysteine, are isopropyl chloroformate,[52] bis(*p*-methoxy-phenyl) selenoxide,[53] and ethyl chloroformate.[54]

3.1.3.3 Other Techniques

Some other techniques used in selenium speciation are HPLC coupled to atomic fluorescence spectrometry with hydride generation (HPLC-HG-AFS), HPLC-electrospray tandem MS, capillary electrophoresis with UV-detection (CE-UV), and CE-atomic emission spectrometry with hydride generation (CE- HG-ICP-AES).[1] Hydride generation is a sample introduction technique that assists in getting nonvolatile species into the gas phase, and is capable of efficient matrix separation and analyte transport, and high selectivity.[55,56] Gómez-Ariza et al.[57] recently demonstrated that chiral separation of selenomethionine in breast and infant milk was possible in under ninety minutes using high performance liquid chromatography followed by microwave assisted digestion and hydride generation atomic fluorescence spectrometry (HPLC-MAD-HG-AFS). Ogra et al.[58] identified γ-glutamyl(methylseleno)cysteine, as well as four other unidentified sulfur-containing species, in garlic and shallot by electrospray ionization-tandem mass spectrometry (ESI-MS/MS), following HPLC-ICP-MS. Capillary electrophoresis offers the advantage of ultra-high resolution and minimal sample dilution and consumption;[34] Sun et al.[59] separated several cationic and anionic arsenic and selenium species, simultaneously, by employing capillary electrophoresis with direct UV detection (CE-UV). Deng et al.[60] demonstrated they could achieve low detection limits for inorganic

selenium species in river and tap water samples using CE-HG-ICP-AES; the HG, serving as an interface between the two, eliminated the need for a nebulization step.

3.1.4 Selenium Metabolism and Bioavailability

Bioavailability is defined as "the fraction of ingested nutrient that is utilized for normal physiological functions," although, as there are no direct ways to measure bioavailability, it can be indirectly measured by the absorption and retention of the nutrient.[61] However, these indirect measurements "cannot address functional bioavailability which is that most likely to be relevant to health."[8]

Regarding food, much less is known about the forms of selenium derived from animals than is known about selenium species derived from plants; selenium in plants is assumed to closely follow sulfur pathways.[8] In order to determine which selenium compounds are in our foods, it is necessary to identify and understand the biosynthetic pathways and metabolism in plants and animals, respectively. Currently, selenium pathways in humans must be inferred largely from rodent models.[8] This information has been comprehensively summarized by Rayman et al.[8] in 2008: although variation is observed between plant species which are categorized as either "accumulators" or "non-accumulators", the major selenium species in plants are selenate, selenomethionine, *Se*-(methylseleno)cysteine, and γ–glutamyl-*Se*-(methylseleno)cysteine. Plants do not typically contain many selenoproteins, and so therefore, also do not have much selenocysteine, whereas animal food sources are abundant in both.[14] In animals, selenotrisulfide, glutathione selenopersulfide, and metallic selenides have been reported in tissue by Burk.[62] More recently, other species identified in animal tissue depend on

whether the source of ingested selenium is inorganic, in which case the major component found is selenocysteine, or the source is of plant origin, in which case selenomethionine is also present.[8] Studies also suggest that selenium from fish is much less bioavailable than other sources of selenium, although, not only has little work been done to speciate selenium in fish, but also the few studies which have been performed show a wide range of compounds and concentrations.[8] Recently, however, Yamashita et al.[63] discovered a new species of selenium distributed throughout the blood and tissues of both bluefin tuna and mackerel; this new compound, which they named *selenoneine*, was further determined to be the predominant organic form in the blood of bluefin tuna. Not many studies on inorganic selenium in food, of plant or animal origin, exist at present.[14]

Besides the importance of knowing the chemical form administered (termed a *horizontal* approach by Gammelgaard, et al.[14]), another major challenge in investigating the bioavailability of selenocompounds in the human body is what happens to them (i.e. how the species are changed) during the physical and chemical processes of metabolism (termed a *vertical* approach[14]); presumably, the chemical form upon gastrointestinal absorption is what will determine its biological fate.[21] It is important to be able to extract these compounds easily and determine if they are stable in highly acidic environments, such as the stomach. It is known that approximately 70-100% of ingested selenium is absorbed by the intestines, whereas 50-75% is excreted through urine, feces, and breath.[21] Of the total selenium present in blood, about 75% is present in the plasma.[21] Dosage, chemical form, and matrix are all factors that influence the bioavailability of selenium in humans,[21] and it is important to investigate this further. Knowing how chemical form in foods can change upon cooking[21] is also an important subject to be explored.

3.2 Research Overview

Selenium, being an essential nutrient, is very important to human health. Despite the controversial results of the recent clinical trials, it is evident that much more work needs to be done in order to better understand the role of selenium and its relationship to cancer. The chemical form of Se should also be scrutinized when supplementing, as some species are considered more toxic than others. There are currently no FDA regulations governing dietary supplements, and as such, many Se supplements sold worldwide have little information available regarding the Se species used and their respective quantities.[64] Selenized yeast, for instance, contains multiple forms of Se and varies widely from batch to batch.[8] Furthermore, of those supplements that do contain ingredient information, many are often incorrect; this disparity could be due to lack of capability to accurately perform such analyses, or it could be that the Se compounds are undergoing speciation changes during shipment and storage.[16] It is therefore of importance to have a method with which to perform accurate, quantitative speciation analysis of these supplements. Many techniques exist for the separation of Se compounds, but few are capable of separating both organic and inorganic species simultaneously.

A new liquid chromatographic method for the simultaneous speciation of inorganic Se and Se amino acids using isocratic elution is presented. The proposed method is capable of separating six selenium-containing species in under 7 minutes using a 4 mM carbonate buffer at pH 9.80 with the addition of 2% MeOH. This is the first time that dimethyldiselenide has been detected simultaneously with selenoamino acids by anion-exchange chromatography. This method also reveals that, despite being a

challenge for this method, selenocystine displays very interesting behavior when mixed with the high pH mobile phase. Quantification was accomplished using calibration with standards and the method was applied to commercial dietary supplements, known to contain Se, as well as to two reference materials, NIST 1568a rice flour and CCQM-K60 selenised wheat flour.

3.3 Experimental

3.3.1 Instrumentation

Initial results were performed using an Applied Biosystems model 400 HPLC pump (PerkinElmer, Shelton, CT) and Rheodyne injection valve model 7125 (Cotati, CA) to introduce mobile phase and sample into a PerkinElmer SCIEX (Ontario, Canada) ELAN 6100 plasma-source mass spectrometer for detection. Later experiments were performed using a PerkinElmer Series 410 LC Pump and Rheodyne valve model 7010. Anion-exchange columns used were a Dionex IonPac AS-11 (Sunnyvale, CA) and a Hamilton PRP-X100 (Reno, NV). The AS-11 column was used in conjunction with an AG-11 guard column, which was replaced with an AG-10 guard column after the AG-11 developed a blockage. A Fisher Scientific (Pittsburgh, PA) Centrific Model 225 Benchtop Centrifuge was available for sample preparation, while a CEM Corporation (Matthews, NC) MARSXpress microwave system, Model 230/6 with Teflon vessels, was used for preparation of samples for total Se determination. A Burrell Scientific Wrist Action Shaker model 75 (Pittsburgh, PA) assisted in sample extraction. Samples were filtered through Whatman, Inc. 0.45 µm PES disposable syringe filter devices (Florham Park, NJ), as well as an Amicon 10kDa molecular weight cut-off (MWCO) ultra

centrifugal filter (Millipore, Billerica, MA). Mobile phase solutions were gravity-filtered through a Whatman Limited 18.5 cm Qualitative 1 filter paper and then sonicated in an Astrason ultrasonic cleaner model 7 from Heat Systems Ultrasonics (Farmingdale, NY) for several minutes before use. A W.S. Tyler, Inc. (Mentor, OH) test sieve with a 500 µm mesh was used for initial preparation of the supplements to remove larger particles and outer shell casings, if present. Instrumental conditions and other experimental parameters may be viewed in Table 3.1.

3.3.2 Reagents and Sample Materials

All solutions were prepared using >18 MΩ cm deionized (DI) water from a Barnstead E-pure system (Bedford, MA). HPLC-grade acetonitrile, certified ACS plus nitric acid, hydrochloric acid, hydrogen peroxide (30%), and Optima-grade 0.2 micron filtered methanol were obtained from Fisher Scientific (Fairlawn, NJ). Sodium hydroxide solution (25% w/v) was purchased from Reagents, Inc. (Charlotte, NC), and HPLC-grade hexane was obtained from Burdick & Jackson (Muskegon, MI). Sodium carbonate, anhydrous, was supplied by Fisher Scientific and sodium bicarbonate was purchased from Mallinckrodt Baker, Inc. (Paris, KY), while sodium citrate, dihydrate, was purchased from VWR International LLC (West Chester, PA) and citric acid, anhydrous powder, was from J.T. Baker (Phillipsburg, NJ). Protease Type XIV bacteria from Streptomyces griseus, seleno-DL-cystine ($(SeCys)_2$), Se-(methyl)selenocysteine hydrochloride (SMSC), seleno-DL-methionine (SeMet), sodium selenate decahydrate 99% (Se^{VI}), and sodium selenite 99% (Se^{IV}) were obtained from Sigma-Aldrich Co. (St.

Louis, MO). Dimethyldiselenide 99+% (DMDSe) was supplied by Acros Organics (Thermo Fisher Scientific, NJ).

All dietary supplement samples were purchased at local retail stores or from online retailers. Supplements included: NOW Selenium (Now Foods, Bloomingdale, IL), Solgar Natural Dry E with Yeast Free Selenium (Solgar, Inc., Leonia, NJ), CVS Pharmacy Selenium (CVS Pharmacy, Inc., Woonsocket, RI), GNC Selenium (General Nutrition Corporation, Pittsburgh, PA), PharmAssure Selenium Yeast (PharmAssure, Inc., Phoenix, AZ), CareOne Selenium (American Sales Company, Lancaster, NY), and CVS Daily Multiple for Men (CVS Pharmacy, Inc.). All of the above were purchased recently, and were therefore previously unopened prior to these analyses. It is worth mentioning that the CVS Pharmacy Selenium was purchased recently, although it had already reached its expiration date 10 months prior. The supplement LifeExtension Super Selenium Complex (manufactured by Quality Supplements and Vitamins, Inc., Ft. Lauderdale, FL) was kept refrigerated after being used in the 2009 study by Amoako et al.[65] SelenoPrecise, also opened in 2007 and refrigerated in the interim, was obtained from Cypress Systems, Inc. (Fresno, CA). The NIST SRM 1568a rice flour was purchased from the National Institute of Standards and Technology (Gaithersburg, MD). CCQM-K60 selenised wheat flour, furnished by Heidi Goenaga-Infante, LGC (Teddington, UK), was opened in 2008 and kept refrigerated in the interim.

3.3.3 Analytical Procedure

3.3.3.1 Preparation of Mobile Phase

Approximately 40 mg sodium carbonate, 300 mg sodium bicarbonate, and 20 mL MeOH were diluted to 1 L with DI water to make a 4 mM buffer solution to be used as mobile phase with the AS-11 anion-exchange column. The pH was adjusted to 9.80 with small amounts of 25% NaOH. Preliminary experiments involving the Hamilton PRP-X100 column used a 2-3 mM citrate buffer, consisting of a mixture of sodium citrate and citric acid, at a pH range between 4.8 and 6.

3.3.3.2 Preparation of Standard Solutions

High performance liquid chromatography-ICP-MS standards were prepared by diluting each of the Se compounds (SeMet, SMSC, Se^{IV}, and Se^{VI}) with DI water to approximately 10 µg g^{-1} Se (m/m). Because $(SeCys)_2$, is insoluble in water, it was dissolved in 1 mL 5% HCl before dilution with DI water. Also insoluble in water was DMDSe, which had to be prepared by adding approximately 1 mL HNO_3 to 0.05 g DMDSe before dilution with DI water. From these 10 µg g^{-1} solutions, aliquots were taken from each, added to 50 mL polypropylene centrifuge tubes, and diluted with mobile phase to produce solutions of various concentrations, containing the six Se species of interest. Because chromatographic peaks of $(SeCys)_2$ were usually smaller in comparison to the other species', and because it was sometimes difficult to distinguish from the Se^{IV} or SeMet/SMSC peaks, $(SeCys)_2$ was only added to the first two standards, whereas the remaining 3-4 standards were prepared without it. Besides that, all standard solutions contained equal concentrations of the different species, with the exception of DMDSe,

which was usually prepared at a concentration 40% less because of the overlap between DMDSe and SeMet/SMSC peaks. Typical calibration standards included 25, 50, 100, 250, 500, and 1000 ng g^{-1} of Se present as SeMet, SMSC, $(SeCys)_2$, Se^{VI}, and Se^{IV}. It was also of importance to prepare the calibration standards on the same day that the samples were diluted in mobile phase (see section 3.3.3.4) in order to ensure that the development of DMDSe and $(SeCys)_2$ within the standards progressed at the same rate as in the samples.

Dilutions of Se^{VI} in 2% HNO_3 were used as calibration standards for the determination of total Se by ICP-MS after microwave digestion. Dilutions of both Se^{VI} and SeMet, prepared in mobile phase, were used as calibration standards for the determination of total Se within the extracted samples by ICP-MS.

3.3.3.3 Sample Preparation and Sampling

Dietary supplement sample preparation varied somewhat, depending on the form in which the supplements were sold. For supplements consisting of a fine powder within a separable casing, the outer casing was removed and the powder was used without further preparation. Harder supplements with a cellulose shell needed to be ground into a fine powder using a mortar and pestle, and then sieved through a 500 μm mesh to remove the outer cellulose layer and larger particles of the supplement.

Approximately 6-12 supplements of each brand were weighed prior to grinding, so that the calculation of Se within the supplements could be performed using the average weight per tablet or capsule. After grinding, the ground supplements were mixed well, to

obtain a more accurate representation of the sample population, before removing small amounts for testing.

3.3.3.4 Enzymatic Extraction

Samples were prepared according to Amoako et al.,[16] with a few modifications. Approximately 200 mg of the ground dietary supplements were added to 15 mL polystyrene centrifuge tubes and mixed with approximately 20 mg Protease XIV and 5 mL DI water and left to shake with a mechanical shaker for approximately 24 hrs. Samples were centrifuged for 30 min at 4000 g, and the supernatant was decanted and filtered first through a 0.45 µm PES filter, and later by a 10kDa MWCO filter at 9500 g for >2 hr. An aliquot between 0.1 and 0.3 g of the clear filtrate was then removed and diluted to approximately 10 mL with mobile phase, and the resulting solution was analyzed by HPLC-ICP-MS. The rice flour SRM was prepared in the same manner; however, because the total Se content (380 ng g^{-1} certified value) was much lower than within the dietary supplements and would also be close to the detection limit within solution, the extract was injected without any final dilution with mobile phase.

Whereas Amoako et al.[16] dried supplements in an oven prior to the extraction process, this step was left out in this study to avoid the accelerated degradation of SeMet to DMDSe, and to avoid the loss of the volatile DMDSe species.

3.3.3.5 Column Storage

Columns were stored in 100% acetonitrile after DI water was pumped through them to remove all remaining traces of sample and mobile phase. It was found that

$(SeCys)_2$ was partially retained on the AS-11 column, and so this procedure was performed, even when the column was going to be used less than 24 hours later, to ensure that all traces of the compound were removed.

3.3.3.6 Microwave-Assisted Digestion

For total Se determination, ground dietary supplements (200 mg) were accurately weighed directly into the microwave vessels with 1.5 mL of concentrated HNO_3 and 1 mL H_2O_2 and were digested according to the conditions shown in Table 3.1. After the samples had cooled, they were diluted to 50 g with DI water and analyzed by ICP-MS against the Se^{VI} calibration curve prepared in 2% HNO_3 mentioned in section 3.3.3.2.

3.3.4 HPLC-ICP-MS Data Analysis

All chromatograms were processed using Chromera software, version 2.1.2.1762 (PerkinElmer, Shelton, CT).

3.4 Method Development

3.4.1 Preliminary Experiments

3.4.1.1 Column Selection and Mobile Phase Optimization

Preliminary experiments began with a procedure following that of Cuderman et al.[46] using the Rheodyne 7125 injection valve. A standard solution containing 100 ng g^{-1} Se in the form of SeMet, SMSC, $(SeCys)_2$, Se^{IV}, and Se^{VI} was injected for all initial experiments. Full separation using a PRP-X100 anion-exchange column (4.1 x 150 mm) with a 3 mM citrate buffer in 2% MeOH at pH 4.8 and a flow rate of 1.0 mL min^{-1},

however, could not be achieved. Improvements were made when the pH was increased to 5.2 and the flow rate was lowered to 0.2 mL min^{-1}, although species still were not baseline-separated.

The PRP-X100 column was replaced by a Dionex AS-11 column. Preliminary experiments using the same conditions as with the PRP-X100 (i.e. 2-3 mM citrate buffer at pH 4.8-6) only afforded 2-3 peaks out of the 5 that were expected (DMDSe had not been added to the method at this time). The citrate buffer was then replaced by carbonate buffer, and the optimal concentration of mobile phase, pH, and flow rate were investigated. As many of the organic Se compounds have similar pK_a values (see Figure 3.1[66]), full separation could only be achieved at high pH, which was possible because the AS-11 column is capable of operating between a pH range of 0-14. Initial experiments were run using a 2 mM carbonate buffer in 2% MeOH with a flow rate of 1.5 mL min^{-1} and a pH of 10.5.

Problems with these harsh conditions arose several months later, marked by a loss of intensity and resolution, and leaks within the injector valve; it was surmised that the Vespel rotor seal within the Rheodyne 7125 valve had degraded because it was not resistant to such high pH values. The resulting poor resolution made it difficult to distinguish DMDSe from the SeMet/SMSC peak, causing all three species to appear as one. Therefore, it was necessary to switch to a Rheodyne 7010 injection valve, equipped with a Tefzel rotor seal that is resistant to high pH. During this time, the 400 series pump was replaced with the 410 series, and the AG-11 guard column was also replaced with the AG-10 guard column. Minor adjustments to the operating conditions at this point resulted in optimum mobile phase conditions of 4 mM carbonate buffer with 2% MeOH

at pH 9.80, while still maintaining a flow rate of 1.5 mL min^{-1}. This allowed all species to elute in less than 7 minutes.

3.4.1.2 Preparation of Stock Solutions

The preparation of HPLC stock standards solutions, with and without the addition of 2% HNO_3, was investigated.

3.4.1.2.1 Preparation of Dimethyldiselenide Standard

Early chromatograms showed a small peak around 1.0 min, immediately before the elution of the large SeMet/SMSC peak. Prior to intentionally adding DMDSe to the standard solution, this peak was typically absent when standards were run the day of preparation, but would emerge after the standard solution was left for a few days. Although this peak was at first unidentified, it was surmised that it was a degradation product of the organic Se species, as it was seen to become more prominent as the standards aged. This compound was later identified as DMDSe following a series of experiments involving the dissolution of a DMDSe standard in several solvents.

Three solvents were investigated in the preparation of the DMDSe standard: DMDSe (~0.01 mL) was dissolved separately in 1 mL hexane, 1 mL of methanol, and 1 mL nitric acid, followed by subsequent dilution with DI water to produce a solution of ~100 ng g^{-1} Se as DMDSe.

3.4.1.2.2 Preparation of Selenomethionine Selenoxide

Amoako et al.[16] describe a method for preparing selenomethionine selenoxide and so the procedure was repeated here for this study. A solution of 1.0 μg g^{-1} SeMet in 0.1 M HCl was prepared, and 50 μL of H_2O_2 were added to a 500 μL aliquot. The resulting solution was placed in the dark overnight and then diluted with mobile phase the next day before analysis. This solution was also prepared two additional ways, to try to more fully understand the underlying chemical process: in one, only H_2O_2 was added to the SeMet (no HCl), whereas the other experiment used the addition of HCl to SeMet, but no H_2O_2.

3.4.1.3 Rate of Dimethyldiselenide Formation

While preliminary experiments suggested that recently purchased dietary supplements were already showing DMDSe degradation, a concern was raised that the sample preparation method could be accelerating the conversion, thereby leading to false conclusions about the supplements' stability. Several experiments were devised to investigate this matter.

It is known that SeMet and SMSC degrade to DMDSe.[65] Since certain chromatographic calibration standards were prepared from stock solutions that had been made up to 3 months prior, it was necessary to be sure that the individual stock solutions had not already degraded to DMDSe. To test this, two separate solutions, each containing approximately 200 ng g^{-1} Se as SeMet and SMSC were prepared in mobile phase. Both solutions were analyzed the same day that they were prepared, and were repeatedly measured every few days for a month to measure the rate of DMDSe formation.

To investigate whether or not the sample extraction process could be accelerating the DMDSe formation, approximately 5 mg of the SeMet reagent were added to 20 mg of Protease XIV and taken through the extraction process as described in section 3.3.3.4. The percent recovery for the injected SeMet versus the eluted amount was also measured during this experiment.

3.4.1.4 Identification of Selenocystine Peak

Preliminary experiments were marked by a complete absence of a peak for $(SeCys)_2$, even when the 100 ng g^{-1} standard of $(SeCys)_2$ was prepared fresh daily and was injected by itself. It was later determined that $(SeCys)_2$ behaves in an interesting way when at high pH; according to Goessler et al.,[66] the compound at high pH should possess double negative charges (see Figure 3.1). This species would appear to be partially-retained on the column when in this conformation, and, in later experiments, rather than an absence of a peak on day 1, there was instead a small, broad peak eluting between Se^{IV} and Se^{VI}. As time went on, this peak would diminish while another peak would begin to emerge between SeMet/SMSC and Se^{IV}. To understand the behavior of $(SeCys)_2$ with this method, a standard containing approximately 1000 ng g^{-1} Se as $(SeCys)_2$, by itself, was injected and monitored over a 40 day period.

3.4.1.5 Enzyme Purity

A study by Cuderman and Stibilj[67] reported potential selenium contamination in enzymes, commonly used for selenium speciation studies. Although they found that β-amylase from barley contained the most amount of Se, they also report finding between

211 and 378 ng g^{-1} Se within Protease XIV, though it appears to vary according to different lots of the material. To test for any such contamination within the enzyme used in these experiments, the following experiments were carried out. Three aliquots (approximately 200 mg each) of the Protease XIV enzyme, lot number 110M1644V, and one aliquot (200 mg) of lot number 051M1893V, were microwave digested according to section 3.3.3.6 and analyzed by ICP-MS for their total Se content. The quantity of lot number 051M1893V remaining after sample preparation was sufficient only for one replicate. Furthermore, to obtain speciation data on the enzyme, an additional sample containing approximately 200 mg of Protease XIV (lot 051M1893V) was weighed out and put through the same procedure as the samples, as in section 3.3.3.4, except the resulting solution did not undergo any dilution. Instead, this solution was injected into the column as-is to see if any peaks could be seen. The insoluble pellet of this last experiment was also microwave digested for its total Se content so that a mass balance could be calculated.

3.4.2 Limits of Detection

The limits of detection for this method were calculated by two different methods; calibration with a standard, containing approximately 100 ng g^{-1} of Se for each species, except about 60 ng g^{-1} DMDSe, was analyzed along with three mobile phase blanks spiked simultaneously with approximately 25 ng g^{-1} Se of each species (approximately 15 ng g^{-1} Se as DMDSe). The standard deviation (s) of the estimated concentration based on the peak areas of these spiked blanks was calculated, and the detection limit was calculated as 3s.

The second method of calculating detection limits was performed by visual inspection of the chromatograms after subsequent two-fold dilution until peaks could no longer be distinguished from the baseline.

3.5 Method Validation

The method of microwave-assisted digestion for total Se determination, as well as the method for the extraction and chromatographic determination for speciation analysis, was validated by three materials with known concentrations. The first was NIST SRM 1568a rice flour, certified to contain 380 ± 40 ng g^{-1} total Se. According to two separate studies,[68,69] this reference material contains mostly Se in the form of SeMet, with minor contributions from $(SeCys)_2$, Se^{IV}, and Se^{VI}, although both studies' calculated mass balance did not add up to the certified value. The second reference material was a four-year old bottle of CCQM-K60 selenised wheat flour, with a certified value of 17.3 ± 0.07 mg kg^{-1} total Se and 28.3 ± 0.5 mg kg^{-1} SeMet (or 11.4 mg kg^{-1} Se present as SeMet) based on the participation of multiple laboratories. The identity of the other form(s) of Se making up the remainder of total Se in this material is not known. The third material, SelenoPrecise, was not a reference material, but is certified as containing between 1100 and 1600 μg g^{-1} Se, mainly as SeMet.[70]

Spike recovery experiments were also performed on real samples to verify that the method could provide accurate speciation data in this particular matrix. The sample chosen for this experiment was LifeExtension, as it was found to contain each of the six Se species of interest. This sample material was approximately 3 years old already, as it had been used in the study by Amoako et al.[65] After initial experiments to determine the

approximate concentrations of each species in the material, spikes were added at approximately 20% of the Se species already present in the material. All species of interest were spiked into the samples simultaneously. Initial experiments showed considerable species conversion when spiking was performed prior to the extraction step, and so the decision was made to spike the material *after* the extraction process in order to maintain species stability.

3.6 Enzyme Extraction Efficiency

The enzyme extraction efficiency was investigated by two methods. First, after speciation by HPLC-ICP-MS, the supplement extract solutions were also analyzed by conventional ICP-MS for total Se determination to verify that any differences between the speciated data and the measured total Se content by microwave digestion was a result of inefficient enzyme activity, rather than a problem with the chromatographic method. The total Se within the extract samples was determined by external calibration with standards of either Se^{VI} or SeMet, both prepared in mobile phase, to account for compound-dependent responses.

Additionally, an experiment was performed to estimate the amount of Se that is lost during the sample preparation steps, i.e. the concentration of Se within the insoluble pellet, remaining after the initial centrifugation and decantation of sample supernatant, the amount of Se lost to the 0.45 µm filter disc, and the amount lost to the MWCO filter. The pellet was analyzed for its total Se content by pouring it directly into the microwave vessels with the assistance of the digestion acids (1.5 mL of concentrated HNO_3 and 1 mL H_2O_2). The same microwave program from section 3.3.3.6 was utilized, except the

final solution was diluted to approximately 14 g with DI water and calibrated against Se^{VI} standards prepared in 2% HNO_3.

It was originally intended to microwave digest the used filter paper from the 0.45 µm filter disc in the same vessel as the pellet, but initial attempts to remove the filter paper from the disc were unsuccessful. It was also deemed too difficult to quantitatively remove the sample material stuck in the MWCO filter; as a result, and only for this particular experiment, the decanted supernatant forwent filtration by both the 0.45 µm filter and the MWCO filter. Most solutions appeared clear, but a few had small particulate matter within them; these particles were allowed to settle before an aliquot of the extract was taken and diluted to 10 g with mobile phase, per usual, and then analyzed for total Se by ICP-MS, calibrated with Se^{VI} or SeMet standards prepared in mobile phase. It was hypothesized that the extracts from this experiment would have a higher Se content than those extracts prepared according to section 3.3.3.4, because they would not be experiencing any loss to the filtration steps. Therefore, it was hypothesized that the sum of the pellet plus the unfiltered extract in this experiment should yield a recovery near 100%.

3.7 Results and Discussion

3.7.1 Preliminary Experiments

3.7.1.1 Column Selection and Mobile Phase Optimization

Figure 3.2 shows a typical chromatogram run for a standard solution on the AS-11 column under optimal conditions. Mobile phase was a 4 mM carbonate buffer with 2% added methanol, raised to a pH of 9.80, and the flow rate was 1.5 mL min^{-1}. All species

eluted in less than 7 min. It is of note that, despite the high pH, SeMet and SMSC coelute and could not be distinguished. Furthermore, the $(SeCys)_2$ peak appeared in two different places, denoted as $(SeCys)_2$" at 1.9 min and $(SeCys)_2$' at 4.4 min. The exact concentrations of Se for each species, as well as their corresponding peaks areas, were as follows: DMDSe (32.8 ng g^{-1}, 30,361 counts), SeMet/SMSC (67.9 and 60.3 ng g^{-1}, respectively, 149,961 counts), $(SeCys)_2$ (49.4 ng g^{-1}, 46,517 counts as the sum of both peaks), Se^{IV} (48.5 ng g^{-1}, 67,349 counts), and Se^{VI} (54.1 ng g^{-1}, 65,477 counts). It was not believed that compound-dependent responses were present, although it appeared that $(SeCys)_2$ was partially retained on the column.

3.7.1.2 Preparation of Stock Solutions

It was determined early on in the development of this method that nitric acid should not be added to the HPLC stock standard solutions. Figure 3.3a shows a comparison of a standard containing approximately 100 ng g^{-1} of SeMet, SMSC, $(SeCys)_2$, Se^{IV}, and Se^{VI}, prepared from stock solutions that did not contain nitric acid. A similar standard, containing equal concentrations of each species but prepared from stocks containing 2% HNO_3, is shown in Figure 3.3b. It is clear that the presence of HNO_3 causes species transformation and alters peak shape, height, and retention time. The Se^{IV} species appears to be oxidized to Se^{VI}, and the peak for $(SeCys)_2$ is absent in the second chromatogram. Whereas the total sum of all the species in Figure 3.3a is 454 ng g^{-1}, the total sum is only 300 ng g^{-1} in Figure 3.3b. It was therefore decided to prepare all stock solutions without acid.

3.7.1.2.1 Preparation of Dimethyldiselenide Standard

Hexane, methanol, and nitric acid all proved suitable at solubilizing DMDSe. Only a slight peak, however, was observed in the hexane chromatogram, whereas no signal was seen for the solution prepared with methanol. On the other hand, the mixture containing HNO_3 produced a very large signal at the same retention time believed to correspond to the supposed DMDSe peak, and so the identity of that peak was confirmed. Nitric acid was chosen as a possible solvent based on the work of Winkel et al.,[71] in which the acid was used to trap the volatile DMDSe and derivitize it to the less-volatile methylseleninic acid. Although this work did not use cryogenic trapping, the nitric acid still proved sufficient at preserving the DMDSe species; it was also postulated that the high pH of the mobile phase could be hydrolyzing the DMDSe into methylselenol. All subsequent stock standards of DMDSe were prepared using nitric acid.

3.7.1.2.2 Preparation of Selenomethionine Selenoxide

The chromatographic results of the solution, prepared according to Amoako et al.,[65] show a peak with an identical retention time to that of DMDSe. It is not known whether this resulting compound is truly selenomethionine selenoxide, as Amoako et al. suggest, or if the addition of H_2O_2 to SeMet is merely accelerating the degradation of SeMet to DMDSe; Amoako et al.[65] did not use molecular mass spectral techniques to verify the identity of this compound, and so it is possible that they were misidentifying this peak as selenomethionine selenoxide, when in fact it was really DMDSe. Furthermore, in their 2007 publication, Amoako et al.[16] demonstrated that the peak, that they identified as selenomethionine selenoxide, grew as the SelenoPrecise sample was

heated; based on findings within this study, it is possible that the peak might really have been DMDSe, which was growing as the sample degraded. However, it is also possible that, since DMDSe appears to elute within the solvent front, a molecule such as selenomethionine selenoxide may also be mostly unretained and elute in a similar manner. Only a molecular mass spectral technique could clear up this confusion and identify the species as DMDSe, selenomethionine selenoxide, or both.

In an effort to investigate this further, though, the same solution was prepared twice more. The first solution contained the SeMet in HCl, but no H_2O_2 was added, whereas the second solution contained SeMet and H_2O_2, but no HCl. The results of the first solution suggested that the major species present was still SeMet, with only a minor contribution to DMDSe, while the second solution revealed a peak that had converted 100% to either DMDSe or selenomethionine selenoxide. Therefore, it is still plausible that the addition of the strong oxidizer such as H_2O_2 is only degrading the SeMet to DMDSe, as was seen in Figure 3.3b, instead of forming selenomethionine selenoxide. As a result, it was decided not to include selenomethionine selenoxide as part of the suite of compounds for which to analyze.

3.7.1.3 Rate of Dimethyldiselenide Formation

The results of running two separate standards of SeMet and SMSC over several days are shown in Figure 3.4. Figures 3.4a and 3.4e show that no DMDSe was present on day 1 for either SeMet or SMSC, respectively, even though the stock solutions from which they were prepared were 2-3 months old. While it can be seen that both compounds degrade to DMDSe (Figures 3.4b-3.4d and 3.4f-3.4g), it is clear from the

chromatograms that SeMet degrades much faster, and produces a measurable quantity after approximately 10 days, whereas a measurable quantity of DMDSe from SMSC was not seen until about twice that amount of time. Given that these compounds are stable for long periods of time in the stock solutions, which exist at a neutral pH, the degradation to DMDSe must not occur until the species have been exposed to the high pH of 9.80 for a few days, suggesting that any DMDSe observed on day 1 of freshly-prepared dietary supplement samples is a result of degradation within the supplement itself, and not as a result of exposure to the high pH of the mobile phase.

To verify that the enzymatic extraction procedure would not cause acceleration of the degradation of SeMet or SMSC to DMDSe, SeMet was put through the same extraction procedure undergone by the samples. The resulting chromatogram displayed a single peak for SeMet and showed no sign of DMDSe on day 1, suggesting that the preparation procedure was not affecting the species stability or accelerating the degradation. The recovery of this SeMet sample was calculated as the amount of Se as SeMet that eluted from the column as a percent of the amount injected onto the column. The result of the recovery experiment was determined to be 99.3%, suggesting that this method is capable of eluting nearly 100% of SeMet, which is the predominant species in many of the dietary supplement samples.

3.7.1.4 Identification of Selenocystine Peak

As seen in Figure 3.5a, one large peak is observed around 4 min for the injection of the 1000 ng g^{-1} standard of $(SeCys)_2$ on day 1. As the standard ages, this peak shrinks as another peak around 2 min begins to emerge, as seen in Figures 3.5b and 3.5c. More

time-based studies of monitoring the same solution week after week revealed that the earlier peak would continue to grow until it finally reached a maximum after about two weeks, while the later-eluting $(SeCys)_2$ peak would eventually disappear entirely. However, even when the peak at 4 min disappeared and it seemed that the peak at 2 min had reached a maximum, 100% elution of the species was still not observed, as it was partially retained on the column. Figure 3.5d shows the standard run on day 12, and now a slight baseline disturbance can be seen around 1 to 1.5 min; by day 40 (Figure 3.5e), those same $(SeCys)_2$ disturbances are still present, but now also an additional peak has emerged around 3.5 min, which would coincide with the retention time of Se^{IV}, had other species been run simultaneously.

It is clear that this method is not ideally-suited for the quantitative speciation of $(SeCys)_2$, as it behaves very strangely at such high pH. It is hypothesized that either the two peaks around 2 and 4 min correspond to different charged species of the compound, designated L^{2-} and HL^- in the figure by Goessler et al.[66] (Figure 3.1), or the later-eluting peak might be partially eluting as $(SeCys)_2$, whereas the earlier-eluting peak may actually be eluting as selenocysteine, the monomer species, if perhaps the high pH of the matrix eventually cleaves the diselenide bond in two.

Fortunately, $(SeCys)_2$ does not appear to be one of the major constituents of the dietary supplement samples. As its quantitation presents a challenge for this method, in an effort to correct for this problem, some samples, believed to possess $(SeCys)_2$, were re-analyzed again several days after their initial preparation, in order to observe the more-developed, earlier-eluting $(SeCys)_2$ peaks. For this reason, it was also important to prepare calibration standards on the same day as the samples were prepared, so that the

species' elution trends would progress at a similar rate. Furthermore, $(SeCys)_2$ was added to only the first two calibration standards so that its affinity for eluting in multiple places throughout the chromatogram would not interfere with the detection of other species.

3.7.1.5 Enzyme Purity

The results of running the Protease XIV material (lot 051M1893V) by HPLC-ICP-MS yielded a Se concentration of 153.3 ng g^{-1}. The chromatogram (seen in Figure 3.6a) shows a SeMet/SMSC peak very near to the detection limit. It is unclear whether or not other species may be present.

Analysis of the pellet after microwave digestion and ICP-MS determined that the remaining solid residue contained 46.4 ng g^{-1} Se. A mass balance of these two results would suggest a total Se content, within this particular lot of Protease XIV, of approximately 199.7 ng g^{-1}.

The total Se determination of this same lot of enzyme, after microwave digestion, produced a Se concentration of 203.8 ng g^{-1}, which is in good agreement with the mass balance calculation resulting from the sum of the extract and insoluble pellet. More replicates of each of these experiments would have been beneficial, but unfortunately, not enough of the enzyme remained.

The other lot of Protease XIV, lot 110M1644V, yielded variable results of the three replicates after microwave digestion. The average ± standard deviation was 354.8 ± 55.4 ng g^{-1}, and the range of measured concentrations covered 306.6 to 415.3 ng g^{-1}.

These results support the findings by Cuderman and Stibilj.[67] Despite the Se contribution from the enzyme used in these analyses, the contamination was considered negligible for most supplement samples. This is because the concentration of Se within the enzyme was close to the method detection limit. Furthermore, the sample preparation procedure only calls for 20 mg of Protease XIV, and so if the concentration within the enzyme is approximately 200 ng g^{-1}, then only 0.004 µg Se is being added to each sample. The problem would arise only for those samples with concentrations close to the detection limit, such as the NIST 1568a rice flour reference material, having only about 25-180 ng g^{-1} Se more than the Protease material.

3.7.2 Limits of Detection

The resulting limits of detection, calculated based on peak area as 3 times the standard deviation of a low calibration standard, were determined to be 2 ng g^{-1} for DMDSe, 3 ng g^{-1} for the peak containing SeMet and SMSC, 10 ng g^{-1} for $(SeCys)_2$, 3 ng g^{-1} for Se^{IV}, 2 ng g^{-1} for Se^{VI}, and 4 ng g^{-1} for the rare occasion when DMDSe could not be distinguished from the SeMet/SMSC peak and instead had to take the sum of the peak areas of all three species. It is worth mentioning that the standards that were used for this experiment were six days old, and so the $(SeCys)_2$ peak area was a summation of the peaks around 2 and 4 min.

The results of calculating the detection limits based on visual inspection, shown in Figure 3.7, were in close agreement with the calculated values and really demonstrate the minimum concentration required to be able to detect each species, as well as the need for

calculation of a limit of quantitation, as the accurate quantitation of the species near the detection limit is subject to much bias, given the large background noise.

3.7.3 Method Validation

Results of the total Se determinations after microwave digestion may be seen in Table 3.2. The total Se content determined experimentally was in good agreement with the certified value for all three certified materials.

The results of the speciation analysis can also be seen in the same table, and these results, too, were in agreement with the certified value, with the exception of the SelenoPrecise material. The primary species appeared to be SeMet or SMSC, with some minor, unquantifiable contributions from DMDSe, $(SeCys)_2$, Se^{IV}, and possibly some unknown species, as well. For the NIST 1568a rice flour material, even without dilution, the signal was still very close to the detection limit (see Figure 3.6b). As such, only a slight peak for SeMet or SMSC could be seen in the chromatogram, but it was difficult to say with certainty if other species were present. Furthermore, part of the good recovery of this material could be due to the contribution of Se contamination from the Protease XIV. The CCQM-K60 selenised wheat flour material had a greater Se content, and so the speciated results were calculated with greater ease and more confidence. Only a single peak, corresponding to the SeMet/SMSC species, was observed for this material, as well, although unquantifiable baseline disturbances were seen around the retention times corresponding to $(SeCys)_2$ and Se^{VI}.

Initial studies for the spike recovery experiments performed prior to the extraction stage resulted in species transformation; the DMDSe and $(SeCys)_2$ peaks were absent

from the spike material, Se^{IV} was greatly diminished, and Se^{VI} was slightly larger. Originally, it was assumed that the Se^{IV} had perhaps oxidized to Se^{VI}, but further investigation revealed something interesting about the LifeExtension supplement. The ingredients list SeMet, SMSC, Se^{VI}, and selenodiglutathione; when Amoako et al.[65] analyzed this same material 3 years prior, they found SeMet, SMSC, selenomethionine selenoxide, S-(methylseleno)cysteine and Se^{IV}. Selenite was not supposed to be present in the material, and yet it was always very prominent when analyzed in both studies. Selenate, on the other hand, was also found to have a large contribution in this study, whereas Amoako et al.[65] did not detect any. To explain this, it was determined that the method of preparing selenodiglutathione involves mixing Se^{IV} with glutathione, and so it was surmised that, as the supplements aged, the chemical process must have reversed itself, leaving only Se^{IV} present in the supplement, while redox processes must be converting the Se^{IV} to Se^{VI} and vice versa.

After the initial experiments, the spikes were instead added simultaneously to the LifeExtension samples *after* the extraction stage. Results of the average of three spike recovery experiments were as follows (expressed as the average ± standard deviation): DMDSe 106 ± 4.7, SeMet/SMSC 94.9 ± 10.1, Se^{IV} 88.4 ± 5.3, $(SeCys)_2$ 176 ± 407, and Se^{VI} 102 ± 2.5. Because of the difficulties associated with the $(SeCys)_2$ identification and quantification, it was no surprise that the spike recovery experiment for that compound was poor. Selenite was also a little low, and it was suspected that this could be interference as a result of $(SeCys)_2$ being present on both sides of the Se^{IV} peak, or because of the Se^{IV} interconversions to Se^{VI} and selenodigluthathione as mentioned above.

3.7.4 Results of Dietary Supplement Samples

The results of the reference materials and dietary supplement samples may also be viewed in Table 3.2. Comparisons are made between the certified value (or the value written on the label for the supplement samples) to the experimental results of total Se determined by microwave-assisted digestion and ICP-MS, the sum of all Se species found by HPLC-ICP-MS, and the ranges found within the supplements for each species.

In many cases, the experimentally determined total Se matches that of the label, with the exception of the Solgar and CareOne supplements, which were found to contain approximately 50 µg Se less than the value on the label. Results of the men's daily multivitamin were slightly lower than the expected value of 110 µg, but analysis of this material might have been complicated by the presence of so many other vitamins and minerals within the matrix of the supplement.

Results of the total Se, as a summation of the individual species determined by HPLC-ICP-MS, were typically lower than both the certified/label value and the total Se values determined experimentally by microwave digestion ICP-MS. Recovery values were on average between 69 and 83% of the expected amount. The reason for the low recoveries was attributed to poor extraction efficiency; typical recoveries of approximately 80% have been documented in the literature for Se extractions[68,69] although Amoako et al.[65] report efficiencies >90%. Other possible reasons for the low recoveries could be the presence of unknown species, for which no standard materials exist, and the inability to accurately quantify $(SeCys)_2$ using this method. The dietary supplement, CareOne, for example, presented itself as the most problematic brand, having a recovery of only about 15% of the total found by microwave digestion because

it contained several unknown species; the only identifiable species were the two inorganic Se species, which were present at low concentrations.

The labels of the dietary supplements, in terms of the form in which Se was present, were accurate for the most part. Although all samples, listed to contain SeMet, showed various degrees of degradation marked by the presence of the DMDSe peak, the ones that listed selenized yeast as the primary ingredient were true to form, particularly for CareOne, which shows a wide range of different compounds, many of which remain unidentified. One exception, however, was the GNC brand; although its label reports that it contains selenium in the form of selenized yeast, only selenite was found, suggesting that sodium selenite was added to the yeast, but it never actually bound to it.

In the final column displayed in Table 3.2, the listed species found by the chromatographic method are presented as a range that does not necessarily add up to the total Se calculated by mass balance of the chromatographed species. This is because difficulties were encountered earlier on, by which the Chromera software was unable to distinguish the DMDSe or $(SeCys)_2$ peaks from the SeMet/SMSC or Se^{IV} peaks, respectively, in some samples and standards. This was probably a result of the clogging of the AG-11 guard column, which was eventually swapped out for the AG-10 guard column, and the chromatograms thereafter displayed much better resolution. Therefore, the ranges presented here are indicated only for those few samples that could, indeed, be distinguished from adjacent peaks using the software.

3.7.5 Enzyme Extraction Efficiency

To measure the efficiency of the Protease XIV enzyme for the extraction of Se from the samples, the supplement extracts were analyzed again by conventional ICP-MS to verify that the total Se measured within the extract was equivalent to the sum of the individual species determined by HPLC-ICP-MS. If the result of the total Se analysis were greater than the sum of the speciated values, then it could be assumed that the issue of poor recoveries was not due to inefficient extraction, but rather to problems with the column or method as a whole. On the other hand, if the total determination were similar to the summation of the speciated results, then it would be likely that the problem was, in fact, due to poor extraction efficiency.

Initially, sodium selenate standards prepared in mobile phase were used as the calibration standards, but the total Se content found for the majority of species, for whom SeMet was the predominant species, was much lower than what was found within the extracts by HPLC-ICP-MS. This discrepancy was attributed to the presence of compound-dependent responses, whereby the organic Se species have lower instrument signal responses than the inorganic forms. The calibration slope for Se^{VI} was 1678 cps per ng g^{-1}, whereas it was 1502 cps per ng g^{-1} for SeMet. While the selenate calibration curve was still utilized for the few samples that contained mostly inorganic Se, a second calibration curve of SeMet, prepared in mobile phase, was used to calibrate the remaining samples. The results (Table 3.3) show that the total Se within the extracts determined by ICP-MS is comparable to the sum of Se species determined by HPLC-ICP-MS, and so it was assumed that the poor recoveries are mostly due to poor extraction efficiency. Minor variations are likely a result of the presence of unknown Se species and the inability of

this method to accurately quantitate $(SeCys)_2$, as seen in the SelenoPrecise and CareOne samples.

Table 3.4 shows the results of measuring the total Se content within the insoluble pellet and the concentration of Se within the unfiltered extract. It was hypothesized that the extracts from this experiment would have a higher Se content than those extracts prepared according to section 3.3.3.4, because they would not be experiencing any loss to the filtration steps. When comparing the extract information shown in Table 3.4 to the sum of the speciated data in Table 3.2, this appeared to be true for the majority of samples, particularly for SelenoPrecise and CareOne. A measurable quantity of Se was also determined in the insoluble pellet material. As expected, the sum of the pellet and unfiltered extract yielded recoveries within the range and near 100% of the expected total Se measured by ICP-MS (Table 3.2). Recoveries that were 80-90% were believed to be a result of sample loss of the pellet in preparation of the microwave digestion, as well as insufficient homogeneity of the unfiltered extract material, given that some had larger particles within the solution. On the other hand, the Solgar sample experienced only a 77.3% recovery because an appreciable amount of it was unfortunately spilled during preparation.

The results of this experiment suggest there is an approximate loss of between 3-35% within the pellet due to poor enzyme extraction efficiency, while the loss of Se resulting from filtration through the 0.45 μm filter disc and MWCO filter might be as much as 38%.

3.8 Conclusions

Selenium chemistry is a field still requiring a great deal of attention. The different species present in foodstuffs and dietary supplements can have tremendous effects on human health; depending on their chemical form and concentration, some of these selenium species may either induce toxicity or have the potential to reduce the risk of certain cancers.

A new method for the simultaneous separation of both inorganic and organic amino acid Se species using isocratic elution has been developed. All species eluted in less than 7 min and it is also the first time that DMDSe has ever been quantified simultaneously with Se amino acids using anion-exchange chromatography without a prior need of derivitization or trapping. This method was successfully applied to the analysis of several Se-containing dietary supplements and reference materials, and through spike recovery experiments and mass balance, it was determined that the method is mainly limited by the poor efficiency of the extraction step using Protease XIV and loss of analyte during the filtration procedures. Clearly, improvements need to be made in this area. This method was also not very capable of measuring $(SeCys)_2$ with confidence, as the high pH of the mobile phase caused the compound to elute in multiple places, as well as to be partially retained on the column.

The ability to detect and quantify DMDSe in these dietary supplements can be used as a unique tool for monitoring the degradation of these materials. Substantial quantities of the compound were detected, even in supplements that were recently purchased and opened, and so it perhaps strengthens the argument that better labeling and regulations are needed. Evidence suggests that DMDSe might actually be more effective

at cancer prevention, however, as it is closely related to methylselenol, which is considered to be the key metabolite in cancer prevention.[72]

This technique can hopefully be useful when used in conjunction with other forms of liquid chromatography as well as molecular mass spectrometric techniques to assist in the full identification and accurate quantification of Se in materials, and to investigate the bioavailability of these species in human or animal studies.

3.9 Acknowledgments

I would like to express my deepest gratitude to PerkinElmer, Inc. for their financial support, and particularly to Ken Neubauer, Pam Perrone, Mike Stanley, and Dennis Yates for their support and guidance on this project, as well as for donations of equipment and software.

3.10 Tables and Figures

Table 3.1 Instrument conditions and other experimental parameters

HPLC	
Column	Dionex IonPac AS-11 hydroxide-selective anion-exchange (4 mm x 250 mm x 13 µm)
Guard column	Dionex IonPac AG-11 (4 x 50 mm) and Dionex IonPac AG-10 (4 x 50 mm)
Mobile phase composition	4 mM carbonate buffer in 2% MeOH, adjusted to pH 9.80 with NaOH
Flow rate	1.5 mL min^{-1}
Injection loop	20 µL
Pressure limits	0-400 bar
Elan 6100 ICP-mass spectrometer	
RF power	1500 W
Nebulizer gas flow	1.01 L min^{-1}
Nebulizer	GemTip Cross-Flow II
Spray chamber	Scott
Detector mode	Dual mode
Sampler/skimmer cones	Nickel
Scanning mode	Peak hopping
Dwell time	100 ms per amu
Number of sweeps/reading	10
Number of reads/replicate	1
Number of replicates	10
Isotopes monitored	^{78}Se
Chromera settings	500 ms dwell time Pulse detector mode 0.9 points/s sampling rate 596 readings
MARSXpress microwave system	
Vessels	XPress vessels, 75 mL Teflon
Power	1600 W
Percent Power Operation	100%
Ramp time	400 min
Maximum temperature	180 °C
Hold time	20 min
Cool down time	60 min

Table 3.2 Summary of the results of reference materials and dietary supplement samples. [a]All values expressed in µg with the exception of NIST 1568a (ng g^{-1}), CCQM-K60 (mg kg^{-1}), and SelenoPrecise (µg g^{-1}). [b]Confidence Level (CL) expressed as 95% confidence with 2-tail probability. [c]Sum of species in this column do not necessarily add up to values in sum of speciation column because not all runs had sufficient resolution to distinguish DMDSe from SeMet/SMSC or Se^{IV} from $(SeCys)_2$. The table has been broken up to span two pages.

Material	Label/certificate amounts[a] and species	Measured Total Se[a] ± CL[b] [N replicates]	Sum of speciation[a]	Range of Measured Species[c]
NIST 1568a rice flour	380, species not listed	368 ± 21.6 [N=3]	325 ± 46 [N=3]	309-345 ng g^{-1} SeMet/SMSC
CCQM-K60 selenised wheat flour	17.3 (total), 11.4 (SeMet)	17.4 ± 1.26 [N=6]	11.9 ± 1.0 [N=6]	10.7-13.2 mg kg^{-1} SeMet/SMSC +DMDSe
SelenoPrecise High Selenium Yeast	1100-1600 (total), approx. 99% organic (70% *L*- SeMet)	1386 ± 50.5 [N=6]	878 ± 63 [N=9]	745-984 µg g^{-1} SeMet/SMSC +DMDSe
Now Selenium	100, *L* -SeMet (yeast-free)	104 ± 12.7 [N=5]	82.0 ± 11 [N=5]	7.49-26.8 µg DMDSe, 57.8-70.8 µg SeMet/SMSC
Solgar Natural Dry E with Yeast Free Selenium	150, *L* -SeMet	92.2 ± 12.7 [N=5]	81.0 ± 3.1 [N=3]	7.03-39.9 µg DMDSe, 41.2-72.6 µg SeMet/SMSC
GNC Selenium	100, Selenized yeast	104 ± 9.2 [N=8]	69.9 ± 3.4 [N=6]	66.1-77.4 µg SeIV

Table 3.2, ***continued***

Material	Label/certificate amounts[a] and species	Measured Total Se[a] ± CL[b] [N replicates]	Sum of speciation[a]	Range of Measured Species[c]
PharmAssure Selenium Yeast	200, Selenized yeast	206 ± 9.5 [N=3]	155 ± 18 [N=6]	11.1-38.3 µg $(SeCys)_2$, 117-140 µg SeMet/SMSC +DMDSe
CVS Pharmacy Selenium	200, Selenized yeast	204 ± 3.0 [N=3]	160 ± 20 [N=3]	11.2-54.2 µg $(SeCys)_2$, 109-122 µg SeMet/SMSC +DMDSe
CareOne Selenium	200, Selenized yeast	147 ± 11.7 [N=3]	22.0 ± 9.0 [N=6]	7.11-19.8 µg SeIV, 5.77-15.4 µg SeVI
Men's Daily Multivitamin	110, Sodium selenate	76.4 ± 4.57 [N=5]	94.0 ± 13 [N=4]	85.4-103.7 µg SeVI
LifeExtension Super Selenium Complex	200 (total), SeMet (50 µg), SMSC (75 µg), SeVI (50 µg), Selenodiglutathione (25 µg)	229 ± 19.2 [N=5]	164 ± 16.1 [N=3]	25.2-47.0 µg DMDSe, 56.4-68.9 µg SeMet/SMSC, 3.84-7.50 µg $(SeCys)_2$, 19.0-23.2 µg SeIV, 33.6-47.7 µg SeVI

Table 3.3 Comparison of total Se, run by conventional ICP-MS, to the sum of the speciated data by HPLC-ICP-MS, in the dietary supplement extracts. [a]Confidence level (CL) expressed as a 2-tailed probability at the 95% confidence. [b]Total measured after microwave digestion found to be 92 µg. [c]Total measured after microwave digestion found to be 147 µg.

SAMPLE ID	Expected µg (µg g^{-1} for SePrecise)	Total by ICP-MS ± CL[a]	HPLC total ± CL
Now	100	71 ± 9.3	82 ± 11
Solgar	150[b]	73 ± 16	81 ± 3.1
GNC	100	70 ± 6.7	70 ± 3.4
PharmAssure	200	157 ± 33	155 ± 18
SelenoPrecise	1100-1600	1119	878 ± 63
Life Extension	200	130	164 ± 16
Multivitamin	110	78 ± 8.1	94 ± 13
CareOne	200[c]	68 ± 11	22 ± 9.0
CVS	200	175 ± 12	160 ± 20

Table 3.4 Total Se determined in pellets, combined with the amount of Se determined in unfiltered extracts by HPLC-ICP-MS for the purposes of mass balance. [a]Confidence level (CL) expressed as a 2-tailed probability at the 95% confidence. [b]Values taken from total measured Se determined by ICP-MS in Table 3.1. [c]Values calculated by comparing the measured Se in pellet versus the expected total Se determined by ICP-MS. [d]Values calculated by taking the difference between the unfiltered extract and the (filtered) sum of species determined by HPLC-ICP-MS in Table 3.1, as a percentage of the expected total Se determined by ICP-MS. Values for Now and Solgar came out to negative numbers and so were expressed as <0.

	Measured µg in unfiltered extract (µg g^{-1} in SelenoPrecise)	Measured µg in pellet (µg g^{-1} in SelenoPrecise)	Sum of extract and pellet (µg g^{-1} in SelenoPrecise)	Expected total Se ± CL,[a] determined from total Se study[b]	% Recovery	% Loss to pellet[c]	% Loss to filters[d]
SelenoPrecise	1308	91.3	1400	1386 ± 50.5	101	6.6	31
Now	80.4	12.6	93.0	104 ± 12.7	89	12	< 0
Solgar	62.7	8.63	71.3	92.2 ± 12.7	77	9.4	< 0
GNC	71.5	18.3	89.9	104 ± 9.2	86	18	1.6
CVS	187	33.2	220	204 ± 3.0	108	16	13
Multivitamin	97.1	2.04	99.2	76.4 ± 4.57	130	2.7	4.1
CareOne	78.5	51.1	130	147 ± 11.7	88	35	38
Life Extension	173	35.7	209	229 ± 19.2	91	16	4.0
PharmAssure	171	16.5	187	206 ± 9.5	91	8.0	7.7

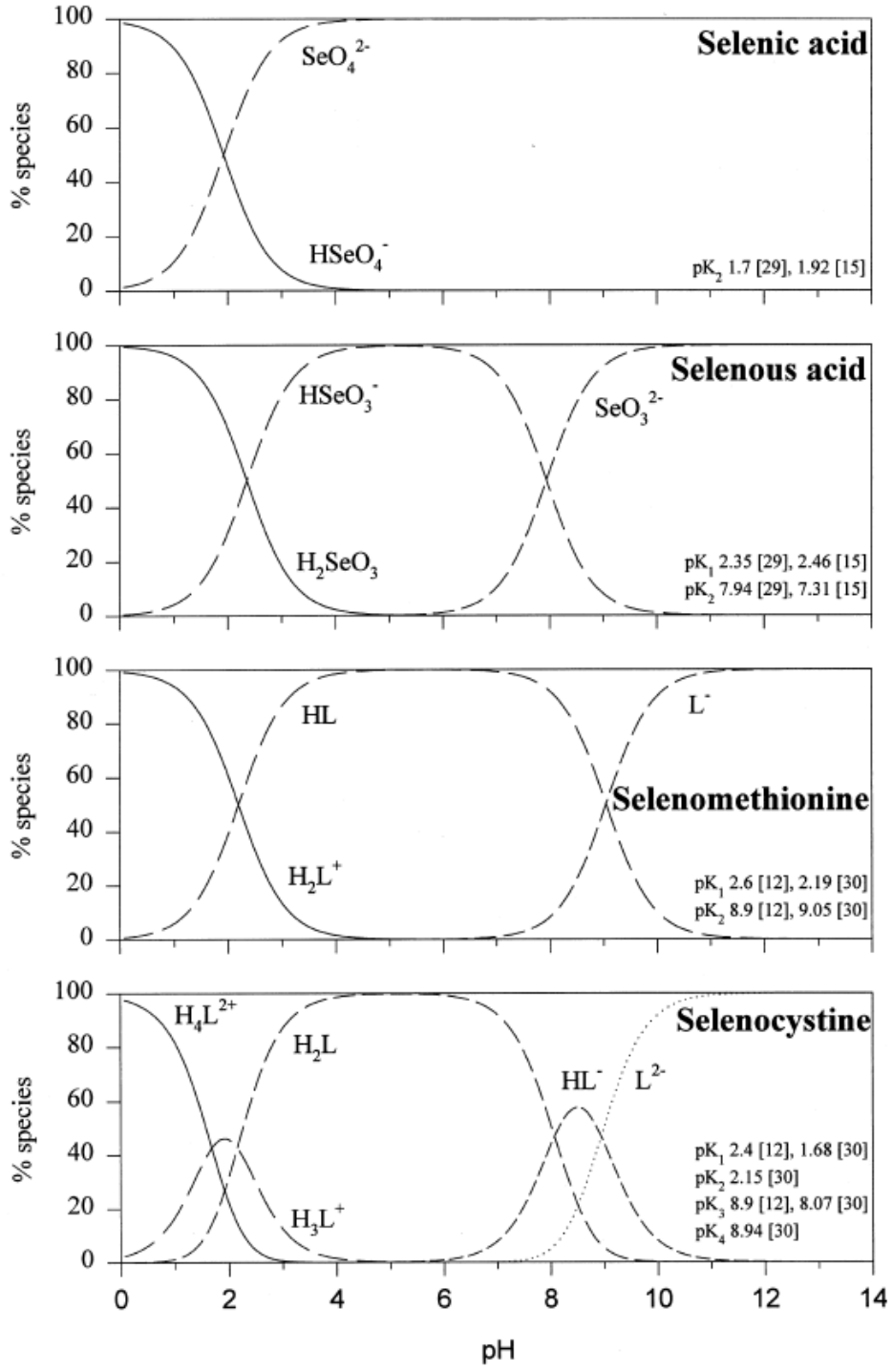

Figure 3.1 Species distribution versus pH.[66]

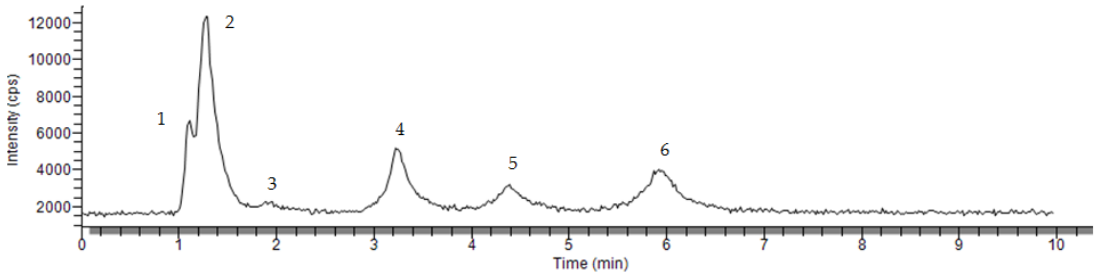

Figure 3.2 Example of a typical chromatogram. Peak identification is as follows: 1 DMDSe, 2 SeMet/SMSC, 3 $(SeCys)_2$'', 4 Se^{IV}, 5 $(SeCys)_2$', 6 Se^{VI}.

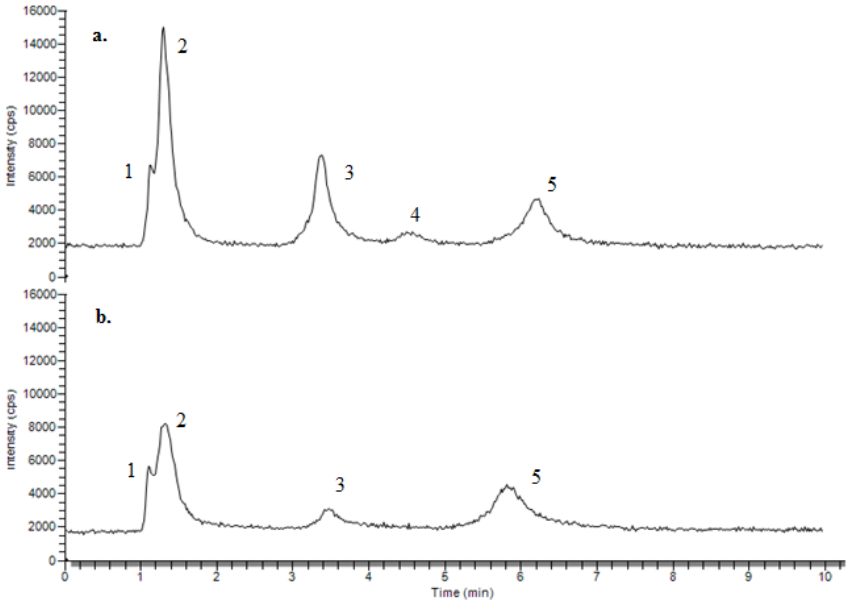

Figure 3.3 Comparison of standard solutions of equal Se concentration for each species, prepared in different matrices. Figure 3.3a sample prepared from stocks diluted in DI water, Figure 3.3b sample prepared from stocks containing 2% HNO_3. Both standards were analyzed immediately after preparation. Total sum of the species was determined to be 454 and 300 ng g^{-1}, respectively. Peak identification is as follows: 1 DMDSe, 2 SeMet/SMSC, 3 Se^{IV}, 4 $(SeCys)_2$', 5 Se^{VI}.

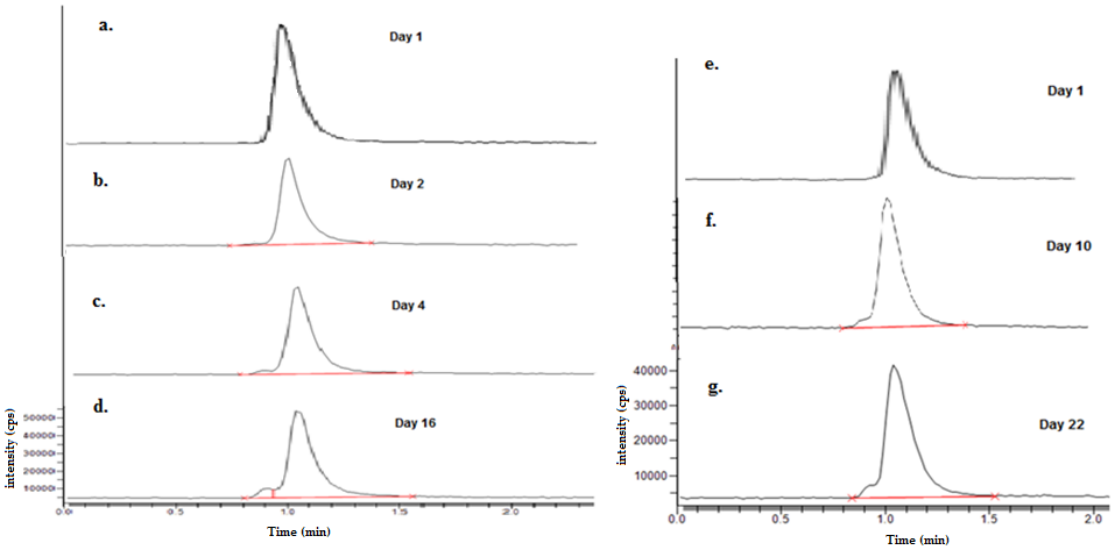

Figure 3.4 Formation of DMDSe over time using a standard of 231 ng g^{-1} SeMet (a-d) and a standard of 200 ng g^{-1} SMSC (e-g).

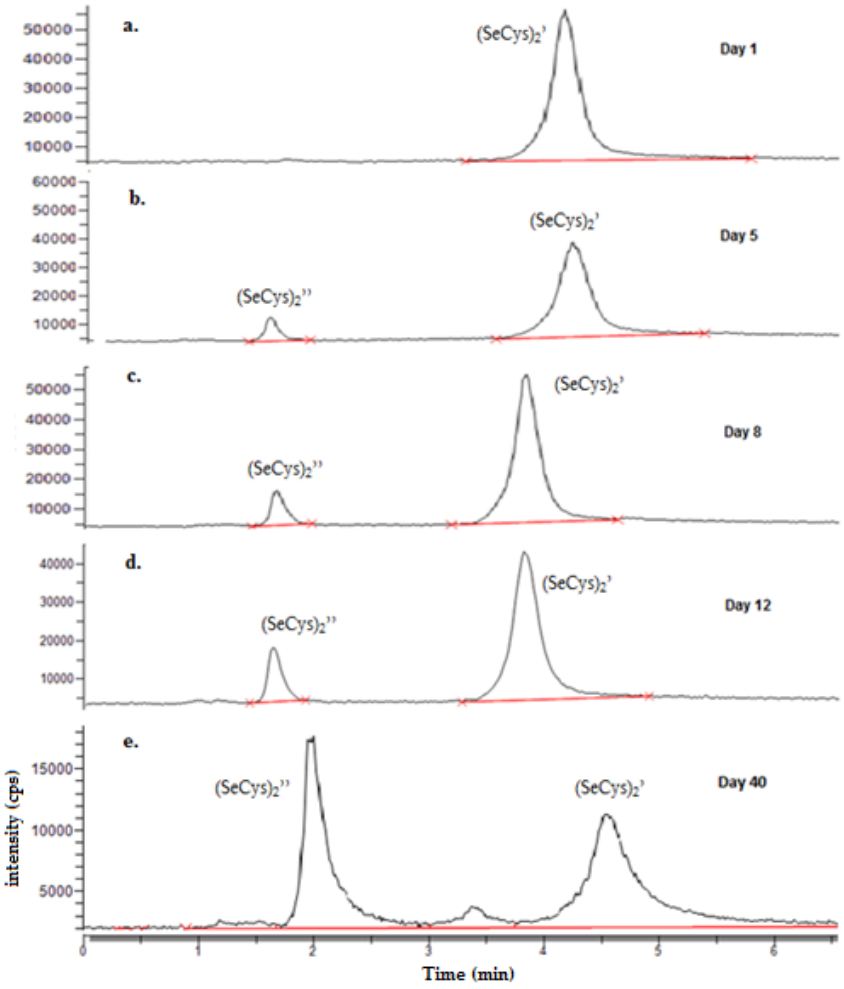

Figure 3.5 Development of $(SeCys)_2$ over time using a standard of 1000 ng g^{-1} $(SeCys)_2$ (a-e).

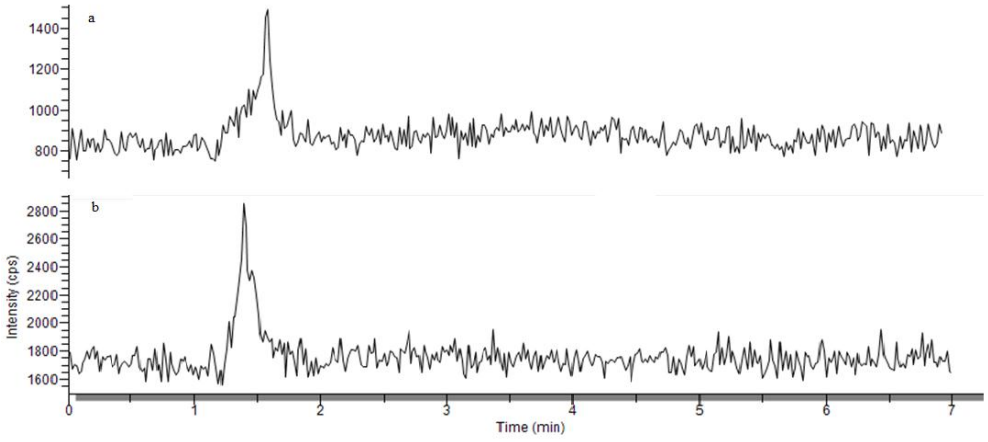

Figure 3.6 Chromatograms of a solution containing approximately 200 mg Protease XIV (a) and 200 mg NIST 1568a rice flour (b).

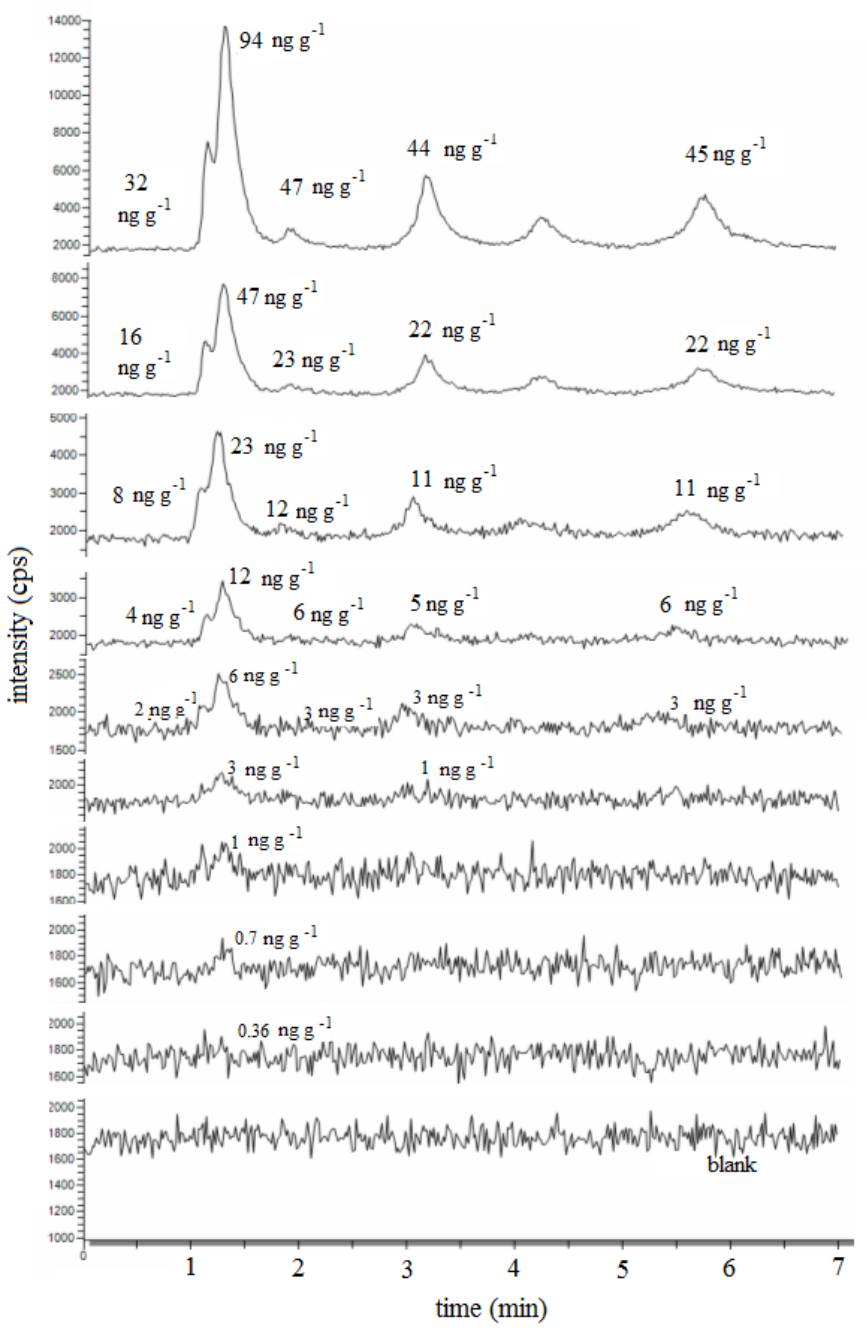

Figure 3.7 Visual estimation of the detection limits for each Se species, after repeated two-fold dilution of a standard.

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CHAPTER 4

TRACE DETERMINATION OF TOTAL MERCURY IN RICE BY CONVENTIONAL INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

4.1 Introduction

Mercury (Hg) and its compounds are highly toxic and are of major concern due to their widespread distribution in the environment. Its elemental forms are readily volatilized and may potentially accumulate in living systems within the food chain.[1-4] The toxicity of mercury depends on its chemical form; the organic forms of mercury, such as methylmercury, are commonly considered more dangerous than the inorganic forms because they are fat-soluble and more easily incorporated into tissues, thus facilitating the uptake of mercury by the organism.[5-7] Furthermore, these methylated forms are also capable of crossing the blood-brain-barrier.[7,8]

Methylmercury has a low volatility and can therefore accumulate in the environment or in living creatures, whereas soluble inorganic mercury, Hg^{2+}, and mercury salts are even less volatile, but can easily be converted by anaerobic bacteria into the more toxic methylated forms, and thus enter into the food chain.[7,8] Mercury sulfide, on the other hand, is typically considered to be the least toxic form of mercury because it is insoluble and stable in the environment.[7,8]

Considering the implications of toxic mercury exposure to humans, especially methylmercury, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives and Contaminants (JECFA) has revised its former provisional tolerable weekly intake (PTWI) of 3.3 $\mu g\ kg^{-1}$ body weight

(bw) of methylmercury to 1.6 μg kg^{-1} bw.[9] For inorganic mercury, the JECFA has set a PTWI of 4 μg kg^{-1} bw, which they consider is also applicable to total mercury exposure from other foods besides fish and shellfish.[10]

4.1.1 The Impact of Mercury on Human Health

Mercury is a dangerous neurotoxin that can damage the brain, kidneys, and lungs, and exposure can also lead to diseases such as acrodynia (pink disease), Hunter-Russell syndrome, and Minamata disease.[11] Although all age groups are at risk, mercury poisoning can be particularly harmful to children in their developing stages, as it can interfere with the ability to learn, delay the development of motor skills, such as walking and communicating, shorten attention span, or in some case, lead to mental retardation.[12,13] In a study by Sakamoto et al.,[14] a strong positive correlation was found between the total mercury in the red blood cells of pregnant women and in the umbilical cord blood of their fetuses; furthermore, the results showed that the concentration of methylmercury in cord blood was 1.6-fold greater than that in the mother's red blood cells, demonstrating the high transport of methylmercury across the placenta and into the developing fetus. Therefore, not only is mercury exposure dangerous to women who are pregnant or nursing, but also, as it is known that mercury can accumulate in the blood over time,[12,15] it is also a danger to women who might become pregnant in the near future, because they can pass the toxin on to the fetus.[16] The EPA estimated in January of 2004 that 630,000 babies in the United States, born between the year of 1999 and 2000 had potentially unsafe levels of mercury in their blood, an indication that mercury contamination is a growing pandemic of national concern.[13] The excretion of mercury

from the body can take anywhere from four months[12] to one year[15] depending on the species of mercury, and provided that additional exposure is not occurring.

Children and adults exposed to mercury may develop problems with the nervous system, including impaired vision, speech, and hearing, tingling or numbness in the extremities, and difficulty walking or writing.[12] Exposure can also lead to mental disturbances, insomnia, dizziness, headaches,[13] and cerebral-palsy-like symptoms.[14] There are two types of mercury exposure: acute and chronic. Acute poisoning occurs when soluble mercury salts are ingested, which can corrode the skin and mucous membranes, or if mercury vapor is inhaled, which can lead to pneumonia or death.[8] Chronic exposure, on the other hand, occurs most often in workers who are exposed to small amounts of mercury on a regular, daily basis,[8] and can lead to many of the symptoms stated above.

Perhaps one of the biggest, documented occurrences of mercury poisoning is the tragedy of Minamata Bay in Japan. As inhabitants of the fishing village, as well as their livestock, began demonstrating neurological problems in 1955, it was later determined in 1959 that the victims were suffering from methylmercury poisoning, later termed *Minamata disease*, from eating contaminated fish from the bay.[16] The source of this pollution was the Chisso Corporation, a large and booming chemical company that produced a wide range of chemicals using a mercury-sulfate catalyst; it was found that this company had been dumping its chemicals, including an estimated 27 tons of mercury-containing byproducts, directly into the Bay from 1932 to 1968.[17]

According to Harada,[16] within the three locations of Minamata Bay considered most affected by the contamination, 220 infants were born between the years of 1955 and

1958; of these 220 infants, thirteen were diagnosed with congenital Minamata disease with a fatality rate of 6.9%. Affected mothers had high levels of mercury in their hair, whereas some samples of umbilical cord blood also had elevated concentrations. Excluding the cases where congenital Minamata disease was diagnosed, the incidence of mental retardation in children born between those same years in Minamata was 29.1%.[16]

Other well-documented outbreaks of mercury poisoning from contaminated food sources include Niigata, Japan in 1965, New Mexico in 1969, and six different locations in Iraq during the 1970s.[16]

4.1.2 Sources of Mercury Pollution

Mercury occurs naturally in the environment, as it arises from volcanic eruptions, crustal degassing, and emissions from forests and bodies of water.[12] However, mercury pollution from anthropogenic sources, such as mining and the burning of waste and coal, is becoming more prevalent and so mercury contamination is most often a result of anthropogenic activities.[12,18] Since the dawn of the industrial age, the amount of mercury pollution in the environment has become increasingly problematic. Thanks to stricter regulations, industries may not be dumping mercury and other waste overtly into the environment, as was the case with the Chisso Company responsible for the Minamata Bay disaster, but despite legislations passed to spread awareness and reduce the amount of contamination, several countries are still observing an increase in emissions,[18] which can have global repercussions. Certain species of mercury can remain in the atmosphere for long periods of time and are known to be transported all over the globe, as evidenced by its presence in the Arctic at concentrations that cannot be attributed to natural or local

sources.[18] Furthermore, thanks to advances in satellite and computer technology, researchers have been able to monitor the movements of individual clouds of mercury and other pollutants across the atmosphere; for example, one study traced "a plume of dirty air from Asia to a point over New England, where analysis of samples revealed that chemicals in it had come from China,"[13] whereas another study traced mercury in rainwater in California back to its origins in China.[19]

There are still other, more discrete ways that mercury pollution may be entering the air we breathe and the food we eat. Mercury and its compounds have been in use since ancient times and historically were used as an anti-microbial agent,[20] and although their use is significantly less, now that the toxicity is well known, they are still used in a wide variety of applications today, such as thermometers, dental amalgams, lighting, vaccinations, cosmetics, mining, and production of caustic soda and chlorine.[21] Pacyna et al.[18] reported in a 2000 review that the primary global sources of mercury emissions are from the production of cements, lead, zinc, pig iron and steel, caustic soda, mercury, gold, and from waste disposal, although by far the largest amount is emitted from the burning of coal. According to an article published in the *Wall Street Journal*,[13] "Scientists long assumed mercury settled into the ground or water soon after it spewed forth as a gas from smokestacks. But using satellites, airplanes and supercomputers, scientists are now tracking air pollution with unprecedented precision, discovering plumes of soot, ozone, sulfates and mercury that drift eastward across oceans and continents."

In fact, scientists estimate that, in the United States, approximately thirty percent of the mercury contamination from air pollution is coming from China.[13] The article also

states that, despite the 600 tons of mercury emitted by China's coal combustion plants each year, many Chinese industries have the option to pay a fee to the government in order to continue operations as usual, rather than investing in ways of reducing pollution.[13] According to the study by Pacyna et al.,[18] mercury emissions have been steadily increasing in Asia between 1990 and 2000, most likely as a result of the growing economy and energy use, whereas Australia, South America, and Africa have demonstrated only a slight increase. On the other hand, mercury emission in North America and Europe has been decreasing due to stricter regulations on emission control equipment and a decrease in the use of combustion for industrial activities.[18]

4.1.3 Mercury Contamination in Food

As evidenced by the disaster in Minamata Bay mentioned above, one of the main ways that mercury is introduced into the diet is through eating contaminated fish and shellfish.[15] Nearly all kinds of fish and shellfish contain mercury,[15] although there are great variations in concentration and in chemical species. Bioaccumulation of mercury in fish is a well-documented problem, and it is for this reason that certain types of fish should be avoided; the toxin can swiftly move up the food chain when contaminated fish are eaten by larger fish, who in turn are eaten by even larger fish and then eventually by humans.[22] Typically, the older and more predatory the fish is, the greater the risk of mercury poisoning as such fish can accumulate significant quantities of mercury into their systems.[22] For instance, fish that should be avoided, particularly by pregnant women, women who intend to become pregnant, and young children, are shark, swordfish, king mackerel, and tilefish, whereas other types, such as haddock, salmon, and

tilapia are generally considered to have less potential for mercury poisoning.[23] It is believed that fish possessing *both* scales and fins contain lower levels of mercury, as their digestive system prevents the absorption of some toxins, whereas fish lacking one or both of the features (such as crustaceans, which lack both, or catfish, which have fins but not scales), naturally absorb toxins in their environment through their skin; this last category is common to bottom-feeders and scavengers.[23]

However, a recent study[24] suggests that mercury exposure through fish may not always be the primary source of mercury poisoning in humans. The researchers determined levels of methylmercury in the inhabitants of four locations within the Guizhou Province of China, chosen because the province is known to suffer from severe mercury contamination, resulting from mining, smelting, and coal burning. The Guizhou Province is not located on the coast, and so perhaps the study is a little skewed because the inhabitants may typically consume less fish than other coastal towns, and the fish they do eat are typically farm-raised and fast growing, and therefore are not given the chance to accumulate much mercury. Despite that, though, the study tested for total and methylmercury in the air, water, and food, and found that rice was the greatest source of methylmercury exposure, containing as much as 9.3 $\mu g\ kg^{-1}$.[24] A similar study found comparable results for the mercury in the rice in the mining district of Wanshan, China.[25] Rice is second only to maize in terms of global consumption, having an approximate annual global production of 600 million tons.[26] It is the staple food of the majority of Asian countries, whose inhabitants typically consume between 200 and 400 g per person per day.[26]

Mercury has poor mobility in soil, and so its uptake by plants most often occurs via the accumulation of airborne gaseous mercury into the plant's tissue;[27] plants can transport both organic and inorganic forms of mercury through the root systems and into the foliage, where it can be reduced to volatile elemental mercury.[27-29] However, microbial activity can also transform inorganic mercury into the methylated forms, thereby creating a potential route for uptake by plants.[30]

A recent study suggests that another common source of mercury exposure might be contaminated high fructose corn syrup (HFCS).[31] High fructose corn syrup was first introduced into the American diet in 1967 and is now used in a wide variety of processed food and beverages more frequently than other sugars.[31] It accounts for over 40% of all caloric sweeteners in today's food and beverages.[32] Many products today feature HFCS as a top ingredient for a number of reasons: it increases the shelf life of foods, retains moisture, and enhances other flavors within the product.[20,31] Furthermore, products containing this ingredient are targeted, because of their sugary properties, specifically to children and, because of their lower cost and greater accessibility than healthier alternatives, to people of lower socioeconomic status.[31] Besides the concern over childhood obesity and other weight-related health risks that have arisen from the increase of HFCS products in the American diet (the Department of Agriculture[33] estimates that the average American consumes 12 teaspoons per day), researchers have found that some of these products may also be contaminated with mercury.[20,31]

Mercury in high fructose corn syrup is allegedly the result of the production process of caustic soda, also known as sodium hydroxide or lye, which is produced in chlor-alkali plants, along with other chemicals such as chlorine, sodium hypochlorite, and

hydrochloric acid;[20,31] caustic soda helps separate the corn starch from its kernels and maintain pH.[31] These chlor-alkali plants use one of two methods: mercury cell technology, which operates by pumping brine through a vat of mercury, or membrane cell technology, which, as the name suggests, uses a membrane instead; more and more companies are transitioning to this membrane cell technology because it is safer and more energy-efficient.[20,31] In 2009, there were approximately fifty mercury cell chlor-alkali plants still operating in the world, four of which were located in the United States;[31] these included the two plants owned by the Olin Corporation in Georgia and Tennessee, a plant in Ohio owned by Ashta Chemicals, and PPG Industries' plant in West Virginia.[31] As of December 2010, the Olin Corporation announced that it was closing its Georgia plant by the end of 2012 and converting its Tennessee plant over to safer technology.[34] According to Dufault et al.,[20] the EPA reported in 2003 that an average of seven tons of mercury was missing from each plant in the year 2000;[35] these plants on average contained approximately fifty-six mercury cells, and each year, they reported to the EPA unaccounted-for mercury losses.[20] More recently, it was reported that Ashta Chemicals lost 415 pounds of mercury in 2009.[36] Dufault et al.[20] and Wallinga et al.,[31] found that, besides losing this mercury to the environment surrounding these plants,[12] mercury was also contaminating the caustic soda products.[20,31] This caustic soda contained anywhere from 0.2 to 1.0 mg L^{-1} of mercury, which could in turn contaminate the food, beverages, and cleaning products that are produced from it.[31,37,38]

Dufault et al.[20] found that 9 out of 20 high fructose corn syrup food and beverage products tested contained measurable amounts of mercury; of these twenty, levels of total mercury ranged from below the detection limit of 0.005 μg g^{-1} to 0.570 μg g^{-1}. In a

follow-up study, Wallinga et al.[31] tested a total of 55 food and beverage products containing HFCS, particularly those items specifically marketed to children, and report that approximately 1 out of 3 of those products contained detectable concentrations of total mercury.

The Corn Refiners Association (CRA), however, retaliated against these findings and list two scientists on their webpage, Dennis J. Paustenbach, Ph.D., of ChemRisk, and Dr. Woodhall Stopford, MD and MSPH, of Duke University Medical Center, both of whom deny the validity of those mercury studies mentioned above.[39,40] The CRA hired ChemRisk as a private consultant to investigate the claims, and although the firm did not repeat these experiments to prove or disprove the results, they instead combed through the two reports and made a long list of reasons as to why the studies performed by Dufault et al. and Wallinga et al. were unsound.[41] Based on a number of reasons, including poor scientific research, "incomplete data and misleading conclusions," deviation from standard operating procedure, and the inability to conclude with certainty that the mercury was not from other sources of contamination, ChemRisk denied the finding that some HFCS products are contaminated with mercury.[41] Also supporting these pronouncements, Dr. Stopford, considered an expert on mercury, was asked to review yet another study on mercury contamination in HFCS foods:[42] Eurofins Central Analytical Laboratory in Metairie, LA, analyzed over 100 samples of HFCS, according to the standard operating procedures used for trace metals, although not necessarily by the optimal methods for mercury determination, i.e. the samples were microwave digested in high pressure vessels with nitric acid and hydrogen peroxide, conditions under which, as observed from the present study, result in mercury loss due to high pressure buildup within,

and venting of, the vessels. The results, reviewed and supported by Dr. Stopford, showed no quantifiable concentrations of mercury in any of the samples.[42]

The U.S. Environmental Protection Agency (EPA) has defined an oral reference dose (RfD) of 0.1 μg kg^{-1} body weight per day of methylmercury;[43,44] the RfD is a term created by the U.S. EPA to suggests a daily intake level below which adverse health risks are not observed during a lifetime.[43] This RfD is meant to apply to children and women of childbearing age, who are considered to be at the most risk, although there has yet to be an RfD for total mercury.[31] Besides for fish and shellfish, the FDA currently has no surveillance program of mercury in other foods or food ingredients manufactured by chlor-alkali plants using mercury technology; at the 77th meeting of the Joint FAO-WHO Expert Committee on Food Additives in 2011, the Committee "noted that there was a lack of quantitative data on methylmercury in non-fish products and on inorganic mercury in general."[10]

4.1.4 Techniques for Mercury Determination

It is well known that rice is an essential food for human beings in most parts of the world.[26] Consequently, environmental contamination by mercury and its compounds, arising mainly from industrial pollution,[12,18] can cause an increase in the toxicity of rice. Therefore, it is of particular interest to accurately analyze and monitor rice samples for their total mercury content. Although a number of techniques exist for the determination of mercury, ICP-MS has become an especially attractive analytical technique for trace elemental detection because of its unique, multi-element capabilities, offering exceptional sensitivity, accuracy, and isotope ratio measurements.[45,46]

Previous studies on the determination of mercury in rice samples include a report from Al-Saleh et al.[47] describing a method for the determination of cadmium, lead, and mercury in rice grain from five different countries by AAS after acid digestion, although the results are largely inconclusive regarding the mercury content in rice because of small sample size and large uncertainty; for example, of the two rice samples tested from the United States, the results ranged from 3.8 to 43.5 μg kg^{-1}, whereas the mean value ± the standard deviation of four rice samples from Thailand was reported as 1.8 ± 1.8 μg kg^{-1}. Lin et al.[48] successfully determined inorganic, methyl, and ethyl mercury compounds by coupling LC to vapor generation ICP-MS after a rapid and simple microwave extraction procedure. Chen et al.[49] utilized flow injection chemical vapor generation with ICP-MS for the determination of arsenic, cadmium, and mercury in cereals and a rice flour reference material using a slurry sampling technique to avoid a dissolution or mineralization step.

There are several challenges associated with the determination of mercury in solid samples by conventional ICP-MS, particularly when the mercury is present at concentrations, such that the concentration in the solution after sample preparation is near that of the instrumental detection limit, as it is in the case with rice grain. One challenge is the choice of sample dissolution technique in which the various mercury species are to be solubilized as the inorganic Hg^{2+} ion.[46,50] However, it is well known that mercury is easily volatilized, so samples cannot be heated to high temperatures without analyte loss and this complicates the use of microwave-assisted digestion and hotplate digestions.[51] Losses equivalent to a few μg kg^{-1} of mercury are of little consequence when dealing with samples containing a few mg kg^{-1}, but that same loss for samples containing only a few

μg kg^{-1} mercury to begin with, is much more serious. Although acid extractions may be performed without heating, they can sometimes be less efficient and more labor-intensive.

The other major problem encountered in the determination of total mercury by conventional ICP-MS is the severe memory effect that results in long washout times, as the mercury adheres to the walls of the sample introduction system.[52,53] Several research groups have proposed procedures to alleviate this problem. Entwistle[54] reported the benefits of offline addition of gold to all standards, samples, and rinse solutions. Mahar et al. have also shown that adding gold as an internal standard improves accuracy and precision,[55] Woller et al.[56] added a surfactant, Triton X-100, and a complexing agent, EDTA, in the analysis of sediments by flow injection ICP-MS. Harrington et al.[57] decreased carryover by adding the sulfur-containing compound 2-mercaptoethanol (2-ME) to the carrier solution in a flow injection system. Several research groups have shown the benefits of the addition of thiols such as 2-ME, dithiothreitol (DTT) and L-cysteine, which are thought to react with mercury via the sulfur atom of the thiol group.[53,58,59] Campbell et al. decreased the memory effect by the addition of salts,[52] but such compounds can deposit onto and clog the cones of the plasma-source mass spectrometer, decreasing the sensitivity.[53]

4.2 Research Overview

Mercury (Hg) and its compounds are highly toxic and are of major concern due to their widespread distribution in the environment. Its elemental forms are readily volatilized and may potentially accumulate in living systems within the food chain.[1-4]

The toxicity of mercury depends on its chemical form; the organic forms of mercury, such as methylmercury, are commonly considered more dangerous than the inorganic forms because they are fat-soluble and more easily incorporated into tissues, thus facilitating the uptake of mercury by the organism.[5-7] Considering the implications of toxic mercury exposure to humans, especially methylmercury, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives and Contaminants (JECFA) has revised its former provisional tolerable weekly intake (PTWI) of 3.3 μg kg^{-1} body weight (bw) of methylmercury to 1.6 μg kg^{-1} bw.[9] For inorganic mercury, the JECFA has set a PTWI of 4 μg kg^{-1} bw, which they consider is also applicable to total mercury exposure from other foods besides fish and shellfish.[10]

There have been a lot of studies surrounding the determination of mercury in fish, and as a result, in the United States, there are many regulations in place to protect people by raising an awareness of the danger;[60] however, unlike several other countries, the U.S. does not regulate mercury in other types of food,[20,61] possibly because the mercury is present in much lower concentrations[62] and tends to be toxic inorganic forms of mercury, rather than toxic methylated mercury.[61]

The majority of mercury studies focus on the determination of mercury in coal, sediment, sludge, and biological materials such as fish and hair, all of which tend to have significantly high concentrations of mercury in the mg kg^{-1} range. Lower detection limits are possible using techniques that are specifically suited for mercury analyses, such as cold vapor atomic absorption spectrometry (CVAAS). There are also a number of dedicated mercury analyzers available. There is no report of the determination of low

concentrations (in the single-digit μg kg^{-1} range) of mercury using conventional ICP-MS, however, and the reason for this is because the measurement of mercury is plagued by severe carryover and memory effects.[52,53] For solid samples, dissolution can also be difficult as mercury is very volatile and is therefore easily lost.[51]

A new method has been developed and validated for the determination of low concentrations (single-digit μg kg^{-1}) of total mercury in rice grain by conventional ICP-MS, in which L-cysteine improved washout and stabilized the signals of both the mercury in solution and of the gold internal standard (added online). Because this new method works with the standard sample introduction system for a plasma-source mass spectrometer, it could be of particular interest to laboratories that have a need for occasional mercury determinations, and as such, may not possess a separate instrument for mercury determinations or a FAST-type system.

4.3 Experimental

4.3.1 Instrumentation

All samples were analyzed with a PerkinElmer SCIEX (Shelton, CT) ELAN 6100 plasma-source mass spectrometer. Samples for the microwave-assisted digestion procedure were prepared in Teflon vessels in conjunction with a CEM Corporation (Matthews, NC) MARSXpress microwave system, Model 230/6. As part of the validation procedures, samples were also analyzed by a PerkinElmer, Inc. (Shelton, CT) SMS 100 automated mercury analyzer, which operates by CVAAS and is capable of analyzing solid samples. A sonicator bath from E/MC Corp., division of RAI Research Co. (Hauppauge, NY) Model 450 Ultrasonic Cleaner and a Fisher Scientific (Pittsburgh,

PA) Centrific Model 225 Benchtop Centrifuge were also available. Rice samples were ground with either a Hamilton Beach Brands, Inc., Custom Grind 15 Cup Coffee Grinder (Washington, NC) or a Krups Fast Touch Coffee Grinder (Millville, NJ). Samples were filtered through Whatman Inc. Puradisc 0.20 μm PES filter media (Florham Park, NJ). Internal standard solution was added online at a T-junction, which simultaneously allowed both internal standard and sample to flow into the T-junction located between the pump and the nebulizer. Instrumental conditions and other experimental parameters are shown in Table 4.1.

4.3.2 Reagents and Sample Materials

All solutions were prepared using >18 MΩ cm deionized (DI) water from a Barnstead E-pure system (Bedford, MA). Certified ACS Plus nitric acid was purchased from Fisher Scientific (Fair Lawn, NJ). Mercury standards were prepared from a PerkinElmer, Inc. (Shelton, CT) 10 mg L^{-1} atomic spectroscopy standard. L-cysteine (97%) was purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Gold for the internal standard was prepared from a Mallinckrodt Baker, Inc. (Phillipsburg, NJ) 1000 mg L^{-1} Baker Instra-Analysed Reagent. Rice flour SRMs NIST 1568 and NIST 1568a, as well as Trace Elements in Spinach Leaves NIST 1570a, were obtained from the National Institute of Standards and Technology (Gaithersburg, MD). Samples of four different brands of rice (two white, two brown) were purchased at local grocery stores.

4.3.3 Preparation of Solutions and Standards

The rinse solution for the plasma-source mass spectrometer contained 1% L-cysteine, to limit the amount of carbon deposition on the cones, and 10% nitric acid, in order to match the approximate acid content of the samples after digestion.

Gold was chosen as the internal standard, as it is similar to mercury in both mass and ionization energy; a 1 μg L^{-1} Au solution was prepared and added online at a T-junction. Spikes were added as 100-200 mg of a 50 μg kg^{-1} stock solution of the aqueous mercury standard to the samples, prior to digestion/extraction. The amounts spiked were selected so that they produced between 2-5 times the observed concentrations of mercury in the sample solutions. The 50 μg kg^{-1} stock solution was also used to prepare all ICP-MS calibration standards (0, 0.25, 0.50, 1.0, and 5.0 μg kg^{-1} Hg). The full range of standards (from 0 to 5.0 μg kg^{-1}) was used when spiked solutions were included in the experiment; otherwise, in the absence of spikes, only standards covering the range 0 to 1.0 μg kg^{-1} were used. All solutions were made fresh daily.

4.3.4 Rice Sample Preparation

Approximately 10 grams of the rice samples were ground for 30-60s into a relatively uniform powder (<500 μm diameter) to ensure representative sampling. Samples were stored in the refrigerator in polypropylene centrifuge tubes until needed. Samples were equilibrated with the laboratory humidity and analyzed "as received."

4.3.5 Data Analysis

All calculations were performed with Microsoft Excel. Calibration functions were fitted by unweighted linear least squares regression. In the case of the microwave-assisted digestion experiments, the average mercury contribution from the reagent blanks was subtracted. This was not necessary for the acid extraction procedure, as the reagent blanks were very close to the calibration blank.

4.3.6 Microwave-assisted Digestion Procedure

Rice samples (500 mg) were accurately weighed directly into the microwave vessels, followed by approximately 200 mg of L-cysteine. Any spikes, prepared from the stock mercury solution were added at this stage. L-cysteine reacts violently with nitric acid, and even more quickly in the presence of organic rice matter, and so this addition was performed in a hood; deionized water (3 mL) and concentrated nitric acid (3 mL) were carefully added, in that order, to lessen the severity of the reaction. After the reaction, the vessels were shaken vigorously and left in the hood overnight, uncapped, to allow for predigestion. Reagent blanks were prepared in the same way. Calibration standards were prepared on the same day as the samples, with approximately 10% nitric acid and 1% L-cysteine; although the standards were not to be digested in the microwave and were instead prepared in polystyrene centrifuge tubes, they, too, were left uncapped in the hood overnight. Samples and calibration standards without L-cysteine were also prepared according to the same procedure.

The following day, the vessels were capped and heated by microwave radiation according to the parameters shown in Table 4.1. This procedure is gentler than most

microwave programs to prevent mercury loss through venting of the vessels, although the samples are not completely dissolved. The samples were diluted to a total mass of 14-15 g with DI water and filtered twice to remove any remaining particles; standards were filtered only once. All samples and standards were analyzed by ICP-MS on the same day. This procedure was validated by the analysis of spikes and rice flour SRM 1568a.

4.3.7 Acid Extraction Procedure

The acid extraction procedure was modified from that of Shao et al.[63] Ground rice samples (500 mg, accurately weighed) were placed into 15 mL polystyrene centrifuge tubes along with approximately 250 mg L-cysteine. Any spikes were added at this stage, and then the extractant mixture was added (5 mL of DI water and 2 mL of concentrated HNO_3). The centrifuge tubes were sonicated in a water bath for 60 min and then centrifuged at 3500 rpm for 5 min. The supernatant was decanted into a 50 mL polypropylene centrifuge tube, and the residue was extracted once more as described above. Afterwards, the two supernatant portions were added together and diluted to 25 mL with DI water to make the final concentration of L-cysteine approximately 1%. Calibration standards were prepared in the same manner. A comparative study prepared samples and calibration standards according to the same procedure, except without L-cysteine. All samples and standards were filtered and then analyzed by ICP-MS on the same day. This procedure was also validated by the analysis of spikes and rice flour SRM 1568a.

4.3.8 Procedure for the Mercury Analyzer

The mercury analyzer was calibrated using varied masses of NIST rice flour SRM 1568a. Ground rice samples (approximately 150 mg) were accurately weighed into the sample boats and analyzed with the program given in Table 4.1. Results were validated with the NIST rice flour SRM 1568, which is a different lot of the same rice flour SRM, as well as by spiking with the rice flour SRM 1568a. The instrument was also calibrated with NIST CRM 1570a, trace elements in spinach leaves.

4.4 Method Development

4.4.1 Preliminary Experiments

Preliminary experiments were performed to optimize the different methods and techniques evaluated in this work. The figure of merit was primarily accuracy of the analysis of the rice SRMs, but results with precisions greater than 10 RSD were considered suboptimal. Experimental factors were considered to be independent, and so the single-cycle alternating variable search was adopted.

4.4.1.1 Minimization of Memory Effect

Several additives to both rinse solutions and samples were evaluated for their ability to minimize the mercury memory effect. The addition of 1-2% NaCl, Au, and L-cysteine was examined.

4.4.1.2 Microwave-assisted Digestion

Preliminary experiments involved an examination of the effect of final dilution volumes, of various microwave temperatures, and of different reagents and reagent concentrations. Sample mass was fixed at 500 mg as recommended by PerkinElmer, Inc. and the CEM Corporation; larger quantities were originally tried, but the experiments resulted in greater difficulty in filtering the samples and more mercury loss due to venting during microwave digestion, as a result of a higher internal pressure.

4.4.1.3 Acid Extraction

Preliminary experiments examined the effect of varying sonication and centrifugation times on the SRM and spike recoveries. For the purpose of consistency with the microwave-assisted digestion procedure, 500 mg of sample were also used for these experiments.

4.4.1.4 Addition of L-cysteine to Samples

The differences in the results between samples prepared both with and without the addition of 1% L-cysteine were examined for the microwave-assisted digestion and acid extraction methods.

4.4.1.5 Mercury Analyzer

Preliminary experiments for the mercury analyzer investigated the role of matrix matching by comparing calibration with aqueous standards with that obtained with solid standards.

4.5 Method Validation

The methods involving microwave-assisted digestion and acid extraction were validated by the analysis of spiked samples, as well as by rice flour SRM 1568a. The spikes were added as aqueous standards to the samples, prior to digestion/extraction, whereas the SRMs were analyzed as if they were rice samples. The spikes were delivered by adding approximately 100-200 mg of a 50 μg kg^{-1} stock solution of the aqueous mercury standard.

Additionally, samples were analyzed by a method involving a different instrumental technique, CVAAS. The performance of this method was also evaluated by the analysis of spiked samples and rice flour SRM 1568. The calibration procedure was investigated: in particular, the differences between results for calibration with aqueous standards and those obtained with solid standards were evaluated.

4.6 Results and Discussion

4.6.1 Minimization of Memory Effect

Of the three additives (NaCl, Au, and L-cysteine) examined for their potential to improve the washout efficiency, only L-cysteine showed noticeable improvements and was, therefore, chosen for the remainder of the experiments.

The wash-in and wash-out profiles of a 10 μg kg^{-1} Hg standard are shown in Figure 4.1 for two different compositions of the sample and rinse solutions normalized to the maximum signal intensity. The Au internal standard was not used in this particular experiment. For solutions containing 2% nitric acid solution, approximately 3.4 min elapsed before steady state was reached (i.e. the signal fluctuated only ± 5%), and 113

min were needed for the Hg signal to return to the original baseline value. When the solutions contained 10% nitric acid and 1% L-cysteine, the times required to achieve steady state and complete washout were significantly decreased to 1.3 min and 13.2 min, respectively.

A signal "spike" was observed at about 1050s for the solution containing 2% nitric acid; this effect was also observed by Li et al.[53] and was explained by the washout of Hg that had been accumulating on the walls of the spray chamber. As has been observed by Jian et al.,[64] the Hg signal for the solution containing 10% nitric acid and 1% L-cysteine was significantly lower than that for the solution containing 2% nitric acid. They argue that the differences between the signals observed for two different concentrations of nitric acid is not related to aspiration rate, nebulizer efficiency, droplet size distribution, or aerosol transport, because the same trend was not observed for Zn or Cd, whereas the opposite trend was seen with As. Although Jian et al. did not mention it, it is possible that the phenomenon could be a result of a decrease in plasma temperature with higher acid concentrations.

4.6.2 Microwave-assisted Digestion Procedure

Initially, after microwave-assisted digestion, the samples were diluted with DI water to a final mass of 50 g, but the resulting mercury concentrations were below the instrumental detection limit. Therefore, samples were diluted only to 14-15 g. Temperatures were programmed to go as high as 190 °C, but analyte loss was always observed at temperatures above 100 °C, which was therefore selected as maximum temperature.

When hydrogen peroxide was added to the nitric acid, the vessels often vented during digestion, giving rise to low recoveries. With greater volumes of concentrated nitric acid (5-6 mL) the acid concentration in the final, diluted solution gave rise to unacceptable accuracy and precision. It was found that digestion with 3 mL nitric acid and 3 mL DI water was able to digest the samples sufficiently to solubilize the mercury (although filtration was required to remove the residual matrix), and it also produced an acid concentration that did not adversely affect the precision of the measurements.

Table 4.2 shows the results of the four rice samples prepared by microwave-assisted digestion. A comparison was made between samples that contained approximately 1% L-cysteine added prior to digestion, versus samples that did not contain L-cysteine. Statistical analysis, using the Student's t-test, indicates that there is no significant difference between the two sample means at the 95% confidence level.

Due to depletion of our stock of brown rice 1, limited data were available for this material. For the samples without the addition of L-cysteine, only an estimate could be made. As the concentrations in the SRMs within that same run were 70% higher than the certified values, the results for the brown rice 1 samples were adjusted accordingly.

4.6.3 Acid Extraction Procedure

The effect of sonication time on (a) the value obtained for the concentration the certified value of the rice flour SRM 1568a (expressed as a percentage of the certificate value) and (b) recoveries of aqueous spikes (both SRM samples that had been spiked prior to the extraction procedure and those that had been spiked after the extraction) is shown in Figure 4.2. The optimum sonication time, defined as all three values within

approximately 80-120%, was 60 min. Similarly, Figure 4.3 shows the effect of varying length of centrifugation time on these same figures of merit; the optimum centrifugation time was around 5 min. Therefore, these optimized times were chosen for subsequent analyses.

The results of the samples prepared by the acid extraction procedure are outlined in Table 4.2. The differences between samples to which L-cysteine had been added and those without L-cysteine were examined. With the exception of white rice 1, all of the results were significantly different according to the t-test at the 95% confidence level. This indicates that the addition of L-cysteine to the samples makes more of a difference with the acid extraction method than it does with the microwave digestion method.

A comparison of the results for the microwave plus L-cysteine procedure with those of the extraction plus L-cysteine procedure by a paired t-test shows that these two methods do not yield significantly different results. However, a comparison of the results for individual samples shows significant differences at the 95% confidence level (though not at the 99.9% level), with the exception of brown rice 1 and SRM 1568a.

The extraction procedure was far more labor-intensive and time-consuming than the microwave procedure; however, despite its drawbacks, the acid extraction method yielded acceptable results for the concentrations of mercury in rice at single-digit, $\mu g\ kg^{-1}$ values, and could be considered to be a viable alternative if a microwave system is not available.

4.6.4 Addition of L-cysteine to Samples

The addition of L-cysteine to the samples improved washout times and may prevent the loss of analyte during the microwave digestion method. The addition of nitric acid to L-cysteine in the presence of organic rice matrix causes (a) the formation of what appears to be colloidal sulfur particles, and (b) gas evolution. Statistical analysis showed that there was no significant difference (at 95% confidence) between the results obtained with the addition of L-cysteine and those obtained without L-cysteine.

The addition of L-cysteine played a greater role in the acid extraction method, as statistical analysis (95% confidence) showed that the results for samples prepared with L-cysteine were significantly higher than the results for those prepared without, with the exception of white rice 1 and SRM 1568a.

Furthermore, the L-cysteine may also contribute to the stabilization of the Au signal, as it was often observed that poor accuracy and precision would arise from fluctuations in the internal standard signal rather than in the mercury signal, particularly when L-cysteine was not present.

4.6.5 Mercury Analyzer

Preliminary experiments involving analysis of rice flour SRM 1568 (certified value 6.0 ± 0.7 μg kg^{-1}) against a calibration with aqueous standards yielded results that were consistently about 50% too high. For instance, the measured value for SRM 1568 was 9.3 ± 0.1 μg kg^{-1} (95% confidence interval). When the mercury analyzer was calibrated with a matrix-matched calibration standard, namely rice flour SRM 1568a (certified value 5.8 ± 0.5 μg kg^{-1}), the results, 6.7 ± 0.1 μg kg^{-1}, were in agreement with

the certified value. Calibrating by this method also yielded spike recoveries (spiked with SRM 1568a rice flour) that were not significantly different from 100%.

A comparison between the slopes of three calibrations is shown in Figure 4.4. The aqueous curve shown in (a) was created by using various masses of a 12 and 100 μg kg^{-1} Hg solution, yielding an unweighted linear least squares regression equation of y = 8742 x + 250, where y is the instrument response in μAbs and x is the Hg mass in units of ng. The calibration curve (b) prepared from the responses to known masses of rice flour SRM 1568a (certified value of 5.8 μg kg^{-1}) was significantly different at y = 12183 x + 165. As an additional comparison, a third curve (c) was created from SRM 1570a trace elements in spinach leaves (certified value of 30 μg kg-1), which produced an equation for the line of y = 10752 x + 183.

4.6.6 Method Validation

The results of the method validation experiments are shown in Table 4.3. For the results of each method, the 95% confidence interval is given for both the values obtained for the concentrations of the rice flour SRMs and the percent recovery of the spikes.

4.6.6.1 Microwave-assisted Digestion

Validation studies for the microwave method were performed using the rice flour SRM 1568a, as well as aqueous mercury spikes that were added prior to digestion. With the exception of the recoveries of spikes into the samples prepared without the addition of L-cysteine, the recoveries were not significantly different from 100%. The values

obtained for the concentration in the SRM were not significantly different from the certificate value.

4.6.6.2 Acid Extraction

For the procedure without the addition of L-cysteine, the spike recoveries were high, but the value for the concentration in the SRM was not significantly different from the certificate value (but only because the uncertainly was relatively high). For the procedure with the addition of L-cysteine, the spike recoveries were not significantly different from 100%, but the value obtained for the concentration in the SRM was low.

4.6.6.3 Mercury Analyzer

The mercury analyzer method was validated by spike recoveries and by the analysis of rice flour SRM 1568. The spike recoveries were not significantly different from 100%, and the concentration measured in the SRM, was in agreement with the certified value.

The results obtained by the methods involving plasma-source mass spectrometry were further validated by the results obtained by the mercury analyzer. The results are shown in Table 4.2 and a comparison of the results obtained by the microwave plus L-cysteine method with the results for the mercury analyzer for the four samples, by a paired t-test, shows no significant difference at the 95% confidence level.

Similarly, a paired t-test shows that there is no significant difference between the set of results obtained by the acid extraction method and the corresponding set obtained by the mercury analyzer.

4.7 Conclusions

A new method of microwave-assisted digestion has been developed for the determination of low concentrations of total mercury in rice grain by conventional ICP-MS. The analysis was made possible with the addition of 1% L-cysteine to all standards, samples, and rinse solutions, as well as the online addition of a gold internal standard. The addition of L-cysteine and gold improved accuracy and precision, while decreasing the memory effect. The L-cysteine also contributed to the stabilization of the gold signal, as well as helping to prevent the mercury from adhering to the sample introduction system. A comparison between microwave digestion and acid extraction showed that the analysis could be performed without a microwave digestion system, though an ultrasonic bath is needed.

Given the current state of understanding of mercury bioavailability and metabolism, it is difficult to say with confidence that the low concentrations of Hg found in the rice samples should not be of much concern. Cleary the amount ingested from the rice samples studied is far below the provisional tolerable weekly intake, even for individuals for whom rice constitutes a large portion of the diet. It is also possible that the mercury is present in forms that can be lost by volatilization during cooking. However, if speciation analyses reveal that the primary form of Hg within rice samples is methylmercury, as was found in a study of rice grown near Chinese industrial facilities,[24] even consuming small amounts of it regularly through the diet might be some cause for alarm, given its cumulative nature within the human body. Children, too, tend to be at higher risk because of the higher consumption per unit of body weight.[66] More studies on the long-term exposure to small amounts of Hg are

perhaps needed, and provisional weekly or daily intake limits need to reflect multiple exposure pathways and different chemical forms.[12,41] It is also necessary to conduct studies on the grain homogeneity per bag of rice so that the variation in mercury content, as well as other trace elements of interest, such as arsenic and selenium, may be estimated.

This new method, which only requires a plasma-source mass spectrometer and standard sample introduction system, may be of particular interest to labs that have a need for occasional mercury determinations, and as such, may not own a separate instrument for mercury determinations or a FAST-type sample introduction system. Furthermore, the method is capable of determining low concentrations that are relevant to the maximum allowable daily or weekly intake levels, and could possibly be applied to the routine monitoring of a variety of foodstuffs (especially other grains and cereals) for the mercury content as well as for a variety of other trace elements of interest, such as arsenic and selenium.

4.8 Acknowledgments

I would like to express my gratitude to PerkinElmer, Inc. for their financial support, and particularly to Laura Thompson for her support and guidance on this project. I would also like to thank PerkinElmer, Inc. for access to the SMS 100 Automated Mercury Analyzer.

4.9 Tables and Figures

Table 4.1 Instrument conditions and other experimental parameters

Elan 6100 ICP-mass spectrometer	
RF power	1500 W
Nebulizer gas flow	1.01 L min^{-1}
Sample flow rate	1.4 mL min^{-1}
Sample pump tubing	Black/black (0.76 mm id)
Internal standard flow rate	0.4 mL min^{-1}
Internal standard pump tubing	Orange/green (0.38 mm id)
Nebulizer	GemTip Cross-Flow II
Spray chamber	Scott
Detector mode	Dual mode
Sampler/skimmer cones	Nickel
Scanning mode	Peak hopping
Dwell time	100 ms per point
Number of sweeps/reading	10
Number of reads/replicate	5
Number of replicates	5
Isotopes monitored	^{202}Hg, ^{197}Au
MARSXpress microwave system	
Vessels	XPress vessels, 75 mL Teflon
Power	400 W
Percent Power Operation	100%
Ramp time	20 min
Maximum temperature	100 °C
Hold time	20 min
Cool down time	60 min
SMS 100 mercury analyzer	
Sample boats	Nickel
Drying temperature	400 °C
Drying time	200 s
Decomposition temperature	800 °C
Decomposition time	200 s
Catalyst temperature	600 °C
Catalyst wait period	60 s
Gold trap temperature	600 °C
Gold trap time	30 s
Measurement time	100 s
Oxidant gas	O_2
Oxidant gas flow	350 mL min^{-1}

Table 4.2 Results for the analysis of rice samples by (a) the microwave-assisted digestion procedure, (b) the acid-extraction procedure, and (c) the mercury analyzer. The results are reported in units of μg kg^{-1}. The observed concentrations of the SRMs are also included; SRM 1568a (certified value 5.8 ± 0.5 μg kg^{-1}) was used for the microwave and extraction procedures, while SRM 1568 (certified value 6.0 ± 0.7 μg kg^{-1}) was used for the mercury analyzer.

Sample ID	Microwave	Extraction	Mercury analyzer
SRM	5.8 ± 0.7 (N = 4)	5.1 ± 0.4 (N = 7)	6.7 ± 0.1 (N = 5)
White rice 1	6.31 ± 1.4 (N = 3)	3.61 ± 1.3 (N = 3)	6.29 ± 0.32 (N = 10)
White rice 2	4.39 ± 0.57 (N = 5)	5.77 ± 0.32 (N = 4)	3.25 ± 0.11 (N = 7)
Brown rice 1	4.06 ± 1.4 (N = 6)	2.86 ± 0.29 (N = 7)	5.95 ± 0.13 (N = 8)
Brown rice 2	4.15 ± 0.25 (N = 5)	5.49 ± 0.42 (N = 5)	3.59 ± 0.11 (N = 7)

Table 4.3 Results of validation experiments for the methods with the ICP mass spectrometer and the method with the mercury analyzer. Values are expressed in μg kg^{-1} together with the 95% confidence interval, except for the SRM, for which the ± term is the 95% confidence interval plus additional allowance for systematic error in the method.

Certified SRM value, μg kg^{-1}	Technique	Observed SRM concentration, μg kg^{-1}	Spike recovery, %
5.8 ± 0.5	Microwave	5.8 ± 0.7 (N = 4)	94.5 ± 16 (N = 4)
5.8 ± 0.5	Extraction	5.1 ± 0.4 (N = 7)	99.3 ± 0.71 (N = 3)
6.0 ± 0.7	SMS 100	6.7 ± 0.1 (N = 5)	103 ± 8.8 (N = 6)

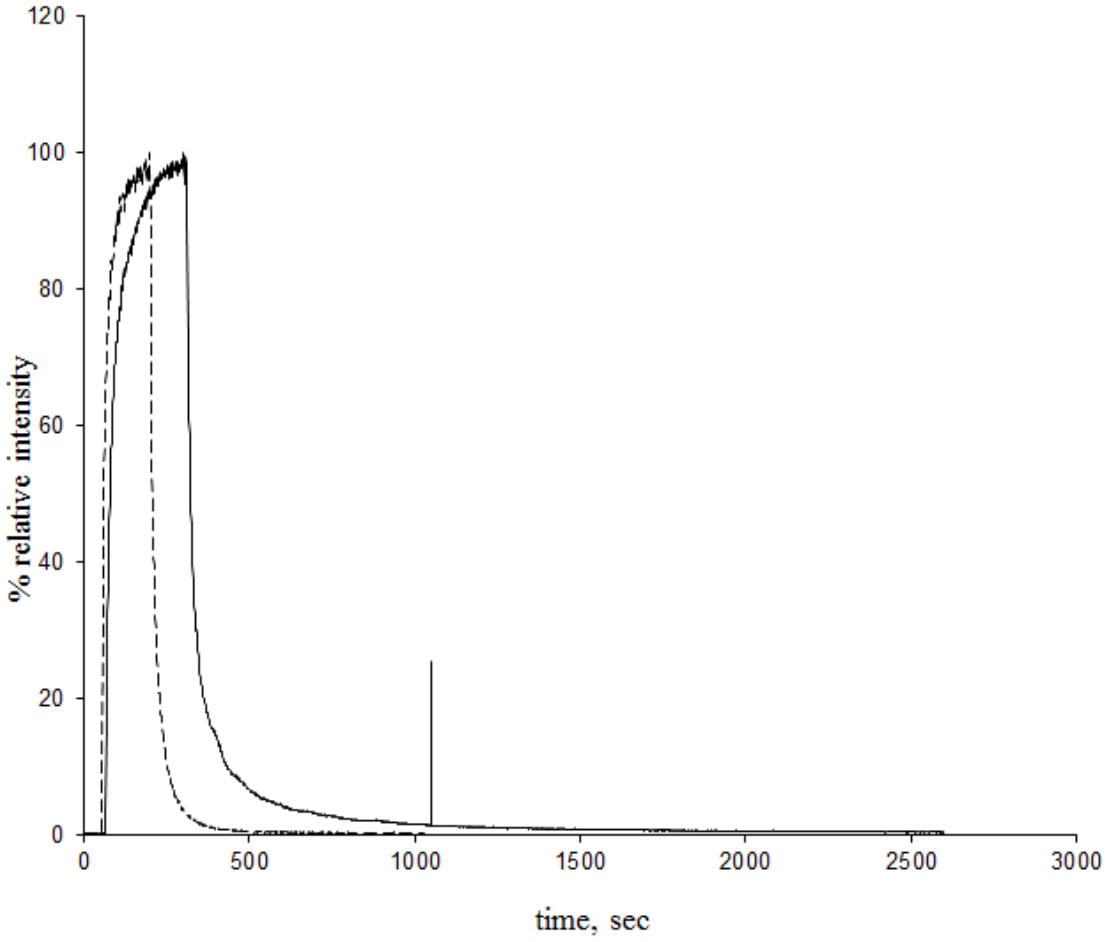

Figure 4.1 Signal responses for wash in and wash out of a 10 μg kg^{-1} Hg standard solution for two rinse solutions: continuous line, 2% HNO_3 solution; dotted line, 10% HNO_3 rinse solution with 1% L-cysteine.

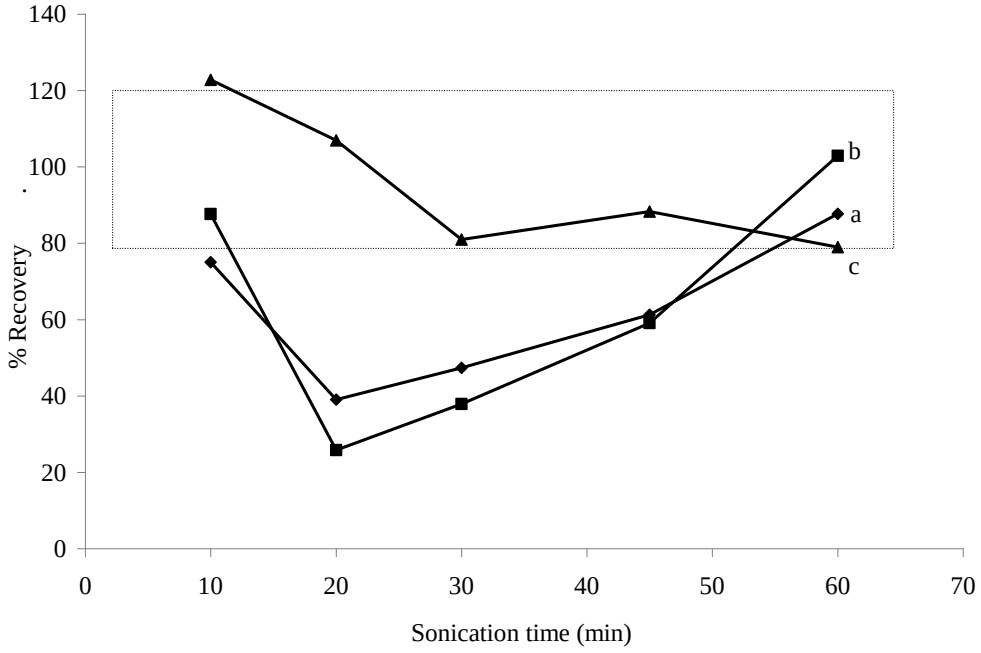

Figure 4.2 Plots of (a) percent of the certificate value of the NIST 1568a Rice Flour SRM and, (b, c) percent recovery of aqueous standards spiked into the SRM as a function of sonication time for the acid extraction procedure. Plot (b) is for spikes added prior to the extraction process; plot (c) is for spikes added after the extraction. The box delineates ± 20%.

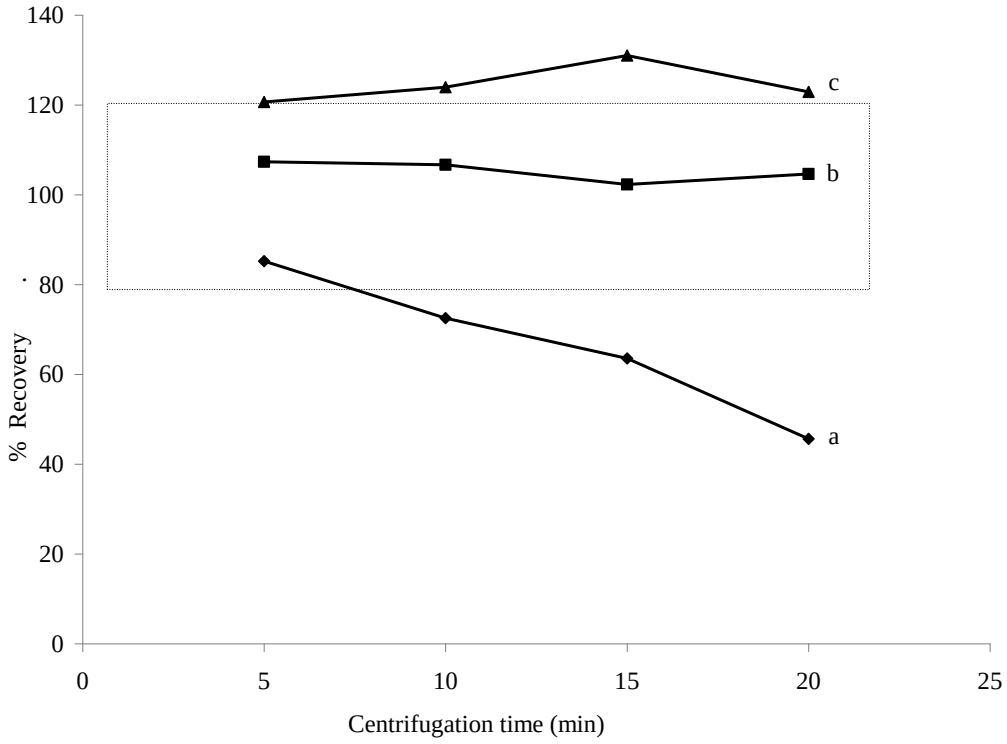

Figure 4.3 Plots of (a) percent of the certificate value of the NIST 1568a Rice Flour SRM and, (b, c) percent recovery of aqueous standards spiked into the SRM as a function of centrifugation time for the acid extraction procedure. Plot (b) is for spikes added prior to the extraction process; plot (c) is for spikes added after the extraction. The box delineates ± 20%.

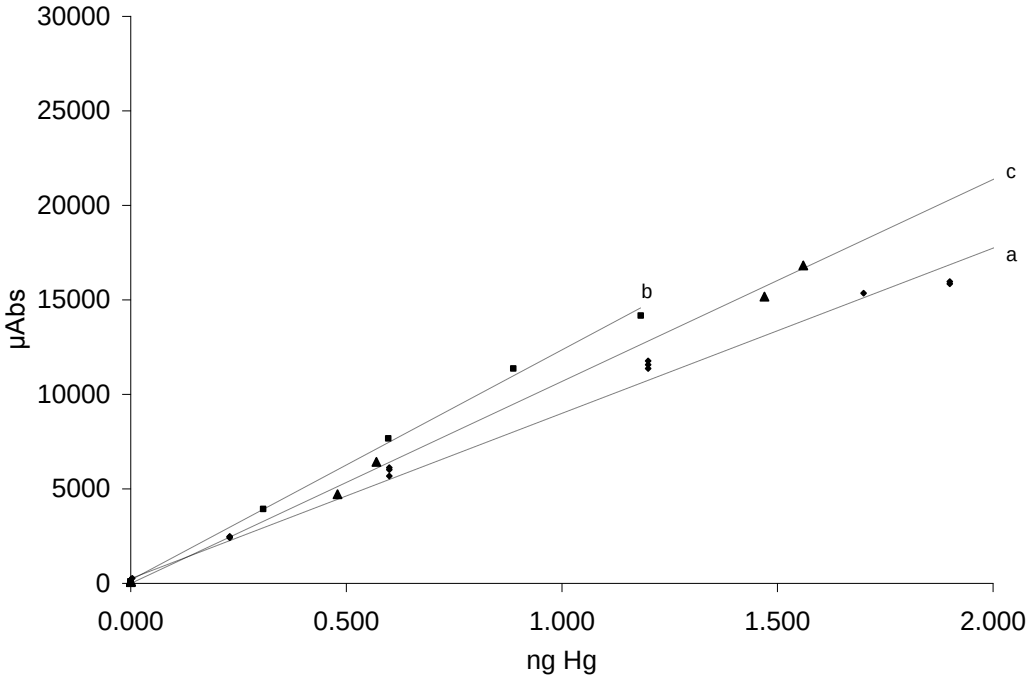

Figure 4.4 Calibration curves for the mercury analyzer based on (a) aqueous standards, (b) rice flour SRM, and (c) spinach leaves SRM. The lines are the best fit by least squares regression analysis. Equations are given in the text.

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CHAPTER 5

DETERMINATION OF RARE EARTH ELEMENTS IN MAYA POTTERY SHERDS

5.1 Introduction

5.1.1 Rare Earth Elements (REEs) in Archaeology

According to Munita et al.,[1] "Chemical analysis of archaeological pottery yields very important information in provenance studies, and much of the information for judging pottery provenience lies in the trace elements." Marques et al.[2] agree that rare earth elements (REEs), in particular, are sometimes critical for fingerprinting these clay materials. A large number of rare earth elements have been used to draw definitive distinctions between pottery samples;[3-6] according to Pillay and Punyadeera,[7] "The chemical similarity of the REE's renders them less susceptible than other groups of elements to mutual fractionation in geochemical processes [i.e. weathering, solubility, erosion, etc.]," and they are typically present as a group within many minerals and silicates in the earth's crust, rather than in the core or mantle. For instance, cerium (Ce) can be used as a geological indication of oxidative conditions, as Ce^{4+}, found in weathered granites, is typically less mobile than the other rare earth elements;[2,8-10] scandium (Sc), on the other hand, typically displays stable geochemical behavior during weathering.[2] However, Marques et al.[2] also note that, "Correlations between pottery and geological materials are difficult to establish, not only due to intra-deposit inhomogeneity and ceramic post-depositional alterations, but also because ceramic manufacture often involves the addition of other materials to the base clay (e.g. other clays or rock fragments as temper)." Furthermore, REEs pose a particular analytical challenge because

of their similar electronic configurations, and hence chemical behavior, leading to stable +3 oxidation states and decreasing ionic radii with increasing atomic number.[7]

5.1.2 Techniques for Analyzing Archaeological Pottery Samples

Instrumental neutron activation analysis (INAA) is the most popular technique for provenance studies of archaeological potteries and clay samples.[2,3,11-13] According to Marques et al.,[2] "The use of analytical methods commonly employed in geological research to study archaeological ceramic materials is not new . . . Chemical analysis in general, and [INAA] in particular, have been used largely in ceramic studies concerned with the provenance of particular kinds of pottery that are found in archaeological contexts but for which the place of origin is unknown. The study of the geological components of ancient ceramics (i.e. the 'clay'), and their comparison with natural clay-rich materials found in the region where the archaeological sites are located, has been seen as a solution to tackle this problem. This approach is particularly useful when no archaeological evidence of pottery production is found, such as kilns and wastes."

Advantages of INAA include high sensitivity and precision for a variety of elements, minimal sample preparation, and it is nondestructive, so it preserves the integrity of the original samples.[3,11] Blackman and Bishop[11] discuss a long-term partnership between the Smithsonian Institute and National Institute of Standards and Technology (NIST), whereby many archaeological ceramics from a variety of different regions and of many different sample types have successfully been analyzed by INAA. The method used standards from the U.S. Geological Survey[14] and the results were validated using a variety of certified standards, such as the NIST SRM 679 Brick Clay

and SRM 1633 Coal Fly Ash. The data yielded very good recoveries of the analytes within the material;[11,15] another material, Ohio Red Clay, was run in conjunction with the SRM and the ceramic samples for further validation. This commercially available material[14] is an air-floated, Redart, 200-mesh clay material supplied by Hammill and Gillespie, Inc. (Livingston, NJ), and although the Ohio Red Clay material has never been certified for its chemical content, it was used primarily to monitor analytical precision.

Although Blackman and Bishop never published the results obtained by INAA for the concentration of analytes within the Ohio Red Clay material, many researchers obtained them through personal communications with Bishop. A 1998 study by Kuleff and Djingova[12] remarks how there is a distinct lack of information regarding the composition of the Ohio Red Clay material, which had become a very important "reference" material used in the analysis of archaeological samples. Kuleff and Djingova pooled the data of ten separate laboratories, performing statistical analysis on the values of 42 elements within the Ohio Red Clay material; nine out of the ten laboratories used INAA, whereas one laboratory employed ICP-OES. The results obtained by INAA were in good agreement with each other, but the optical emission results were in general much lower than the results by neutron activation. Unfortunately, the sample dissolution procedure for the optical emission analysis was not provided in the paper.[12] Munita et al.[1] made a comparison between Bishop's results for the clay, using their own INAA. The study compared the results obtained by three separate neutron activation instruments, each operated under different conditions. The results of the three studies, each measuring the Ohio Red Clay sample, were further compared to the results obtained by Bishop, and were found to be in close agreement with each other. Later, a "reference" material,

designated *New* Ohio Red Clay, became available, and is now also commonly used in the analysis of archaeological ceramic samples. The compositions of Ohio Red Clay and New Ohio Red Clay are very similar, as shown by a comparison by Kuleff;[16] the results for Ohio Red Clay were the ones reported in the 1998 study by Kuleff and Djingova,[12] while the values for New Ohio Red Clay were obtained through a personal communication with Glascock. It is not known how these values were come by.

Instrumental neutron activation analysis, however, also has a number of drawbacks, including cost of operation, analysis times that can take up to several weeks, the need for experienced staff, and limited availability to archaeologists.[3,11] Furthermore, once nuclei have been activated, some of them can remain radioactive for many years, thus requiring certain handling and disposal protocols, and for this reason, many of the world's neutron sources are being decommissioned, which raises the cost of analysis further.[11] It is expected that most will be offline by the year 2050,[16] and so alternative techniques are being explored with urgency.

One alternative technique is X-ray fluorescence (XRF) spectrometry, which has been successfully used in provenance studies to detect major elements as well as few of the minor elements.[17] As a means of identifying clear distinctions between groups of samples, De Vleeschouwer et al.[18] successfully used wavelength-dispersive XRF spectrometry for the determination of major, minor, and trace elements in ceramic sherds and potential clay sources, although they made no mention of whether the technique was also applicable for REE determination. According to Speakman et al.,[19] however, XRF is not well suited for making distinctions between groups of samples; in their study, ceramic samples were analyzed by both portable XRF spectrometry and INAA, and although the

two techniques were in agreement for the elements that were common to both, XRF spectrometry could not determine as many elements as INAA, and it demonstrated poor accuracy and precision for the heterogeneous samples.

Another technique that is becoming quite popular for archaeological pottery samples and geological provenance, though still in its infancy for this particular matrix, is inductively-coupled plasma mass spectrometry (ICP-MS).[16] This technique has a number of advantages, such as small sample sizes, low solution detection limits (sub-part per billion), simultaneous detection of over seventy elements, isotopic ratio measurements, and lower cost and less time per analysis compared to INAA.[6,20] However, like any technique, it also has its drawbacks, and for this particular sample type, these are related to the sample preparation procedure, as the technique is only applicable to solutions with relatively low total dissolved solids content.[17] Alkali fusion is sometimes used, but this creates a large amount of total dissolved solids, thereby making the resulting solutions difficult for analysis by ICP-MS, and also because of the great deal of dilution required, trace elements often end up below the detection limit of the instrument.[20] As a result, acid digestion is considered a more viable alternative,[20] although getting these clay samples into solution can be a significant challenge, and oftentimes dangerous acids, such as hydrofluoric acid (HF), are employed to ensure that all mineral phases dissolve and no insoluble precipitates form.[16] Furthermore, ICP-MS is a destructive technique, and so the analysis of rare and highly-valuable artifacts may not be recommended, as it will irreparably damage the item. Coupling laser ablation to ICP-MS is one way to avoid damage to the samples, but this technique also has its share of limitations. For example, it has higher detection limits (in the parts per million range) than conventional ICP-MS

and it lacks suitable standards. It also is not ideal for bulk samples and it is absolutely critical that the samples be completely homogeneous.[16]

Holmes et al.[20] developed a procedure for the analysis of ceramic samples by ICP-MS in which samples were dissolved in HF and HNO_3. The results were compared to those previously observed by INAA for the same samples, and while there was agreement for the majority of elements, for others, such as Sc (subject to an unknown interference with ICP-MS), Yb, Th, and U (present at concentrations in solution too low to produce a measurable response by ICP-MS), and Cr and Rb (likely due to inefficient sample dissolution) there was poor agreement.

Kennett et al.[3] proposed a new method for the analysis of prehistoric ceramic samples using ICP-MS. Although they admit that HF is very dangerous to work with, they say that, with ICP-MS, it acts as an adequate replacement for INAA, as it serves to break down the silicate phases that are commonly found in ceramics and often can lead to isobaric interferences.[21] They used a Mars Microvap system (CEM, North Carolina), which allowed them to evaporate and neutralize the corrosive acids much faster and more efficiently than when using a hot plate, although evaporation of these acids can present its own problems, too. Formation of insoluble calcium fluorides can actually precipitate out the rare earth elements, thereby leading to poor recoveries. Kennett et al.[3] discuss how some researchers add perchloric acid, another dangerous chemical, to these insoluble precipitates in order to get them into solution again. This can be very dangerous while on a hot plate, however, as it has the potential to explode. Therefore, Kennett et al.[3] proposed, in their newer method, the addition of hyper-saturated, ultra-pure boric acid in order to diminish the formation of calcium fluorides during the evaporation process. The

boric acid complexes with the hydrofluoric acid, thereby allowing everything to go back into solution. The results they obtained by this method were in good agreement with the results obtained by INAA.

Mariet et al.[21] also employed ICP-MS for the analysis of soil and geological samples, but explored the efficacy of using safer, alternative reagents as opposed to hydrofluoric or perchloric acids. In their work, they compared a digestion technique using typical sample dissolution reagents (HNO_3, HF, and $HClO_4$) versus a similar technique utilizing only the safer and less-expensive ammonium fluoride (NH_4F) and HNO_3. The researchers chose to evaporate the solutions to remove the fluorides as volatile SiF_4, rather than add boric acid, which would complicate the matrix and clog the sampling and skimmer cones of the ICP-mass spectrometer because of the high dissolved solids. The results of the two procedures were in good agreement with each other and with the certified values of reference materials, although some issues with isobaric interferences and coprecipitation arose and are discussed.

Hu et al.[22] also used NH_4F as an alternative sample dissolution reagent to HF or $HClO_4$ for the dissolution of geological samples for the determination of several elements in geological samples by ICP-MS. They note that recoveries for several of the rare earth elements were low due to fluoride precipitation, but they report that the addition of HNO_3 between the digestion and the evaporation steps can prevent this. Results were in good agreement with published values.

5.1.3 Maya Civilization in Motul de San José

The Maya civilization occupied the region of Mesoamerica, including parts of present-day Mexico, Guatemala, Belize, Mérida, Honduras, and El Salvador (see map[23] in Figure 5.1). The Maya civilization consisted of several major cities, including Tikal, the most famous and believed to be one of the superpowers, Seibal, Yaxchilan, Machaquila, and Motul de San José,[24-26] although the political relationship between these cities is not fully understood.[24] Motul de San José is located approximately 3 km from the northwest shore of Lake Petén Itzá in central Petén, Guatemala.[23] While archaeological discoveries reveal that the height of Maya civilization in Motul de San José arose in the Late Classic period (*c.a.* 600-830 A.D.), occupation of the area extended from the Middle Pre Classic (600-300 B.C.) to the Early Post Classic (950-1200 A.D.) eras.[27,28] Occupation of the periphery surrounding Motul de San José may even extend farther back in time to 800-700 B.C.[27,29] Approximately 1.4 km^2 of the city, including its city center and 230 remaining structures, are now part of a national park, but the city extends beyond the park borders to encompass an area of approximately 4.2 km^2 in total.[27]

According to hieroglyphic discoveries, Motul de San José is believed to be an important political center[27] and is one of several capitals bearing the Ik' Emblem Glyph;[23,27] this glyph, shaped like the letter 'T' and discovered on the city's monuments and pottery vases, was identified in 1976 by Marcus[30] as a symbol referring to the title of the divine ruler for that particular city.[27] Based on chemical, iconographic, and epigraphic (study of inscriptions) studies, Motul de San José is believed to be one of the main production centers of finely-painted, polychrome vases bearing the Ik' Emblem

Glyph, referred to as the Ik' Style of pottery and ceramics.[23,31-33] These polychrome vessels are believed to have first originated around the 700s A.D.[23]

5.1.4 Scholarly Debate

There is a major debate among scholars regarding the Late Classic Period Maya: was the Maya political structure centralized or decentralized?[24-26,34-40] For example, the relationship between the major city of Tikal and some of the smaller cities, such as Motul de San José, is not known.[24] A centralized government would suggest a hierarchy with an elite class controlling economic infrastructure, production and distribution of goods, whereas a decentralized form of governance would suggests a more autonomous structure, whereby each city is in competition with the next.[23,27] According to Marcus, fluctuations between periods of centralization and decentralization may also have been at play throughout the history of the Maya.[27,41] It is of note that a decentralized model could still possess its own version of an elite hierarchy, and it is believed that Motul de San José was ruled by a succession of kings, considered by the people to be divine rulers.[27] Many of the details surrounding Maya society are known from the interpretation of their hieroglyphs.[23] Early studies of these icons led scholars to believe the focus of these writings was centered on religious, mythical, and cosmological themes, but more recent evidence revealed that these images also detailed the names and images of city officials, elite artisans and scribes, and political events, thus yielding more information into the history of these peoples.[23] Additional analyses of architecture and settlement patterns[23] assist scholars in making informed postulations to reconstruct ancient political systems, but chemical analyses of the artifacts and geological

surroundings, particularly regarding their REE content, can also prove to be a useful tool in gaining sociopolitical understanding. Pottery may have been a form of currency[23,32] to the Maya, and so an understanding of the production, ownership, and distribution of these artifacts might provide evidence to aid in the identification of the relationship between economic controls and political power, thereby bringing us closer to resolving this scholarly debate about centralized versus decentralized political structures.[27]

5.1.5 Relationship of Ik' Style Polychrome Pottery to Politics

According to Halperin and Foias,[23] "Archaeologists have drawn on pottery analyses from a range of theoretical approaches as a means to advance an understanding of political organization and the agency, power dynamics, and social contexts underwriting it. Pottery is often considered as a means of information transmission in that material culture is seen as a reflection of cultural identities, sociopolitical boundaries, and other cultural ideas and structures. Rooted in structuralist theory, artifact styles (and their iconography, if present) are thought to have either actively or passively communicated the sociopolitical organization and meanings of a given society."

That being said, the identification of provenance, or the place of origin, i.e. not just where the artifact is found, but where the raw materials of the artifact originated, becomes a very important key to understanding intra- and inter-regional political practices. Discovery of Ik' Style polychrome pottery in Motul de San José has led to many important findings that have changed the way scholars imagine Maya economic structures; though once believed that only an elite class of people possessed these ornate, polychrome vessels, analysis of the sherds (pottery fragments) reveal that, although the

elite may have possessed more, the elite were not the sole possessors.[23,42-45] Sherds have been found in residences of the elite and non-elite, alike.[23] Apart from their use in ritual feasting, it is now believed that these vessels also served as a form of currency to solidify alliances and create and pay debts,[23,32] thereby potentially making these vessels a symbol of political power. Furthermore, analysis of the vessels' iconography and epigraphy (the study of inscriptions) may also suggest that the production of the pottery was seen as a divine process, thereby bringing prestige to the manufacturer rather than the owner. The concept of a "palace-school" may having existed, as well, whereby elites could participate in the production of these vessels.[23] Evidence of a production site near the royal court suggests the center of the city was an important place of manufacture of this style of pottery.[23] Chemical analysis of these sherds and those from elsewhere in the city has identified different styles[23,32] within the Ik' Style of polychrome pottery, and may help to identify different political spheres or distinct social interactions.

5.1.6 The Motul de San José Archaeological Project

According to Blackman and Bishop,[11] "Unravelling the secrets behind the manufacture and movement of artefacts has long been a core pursuit or archaeological study. However, the secure identification of the source of the materials used to fashion artefacts requires the melding of archaeological information with data obtained from the physical sciences." The Motul de San José Archaeological Project is a long-term, interdisciplinary, and multi-author research program, directed by Dr. Antonia Foias and Dr. Kitty Emery. The goal of the project is to obtain a better understanding of the economic and political structure of the Maya within the ancient city of Motul de San José

during the Late Classic Period (600-900 A.D.).[27] It is believed that the Maya Ik' Style polychrome pottery may have influenced, and have been influenced by, political relations,[5] and so analysis of these vessels may provide a key to solving the debate on centralized versus decentralized society.

Between 1998 and 2005, Dr. Foias and Dr. Emery conducted excavations within, and along the outskirts, of Motul de San José. The excavations sites were divided into five groups, A-E (see Figure 5.2[30]), each consisting of courtyards, residential buildings, and at least one temple-pyramid.[27] The largest group, Group C, included the main plaza and Acropolis, believed by some to be the royal palace and court belonging to the rulers.[27]

The archaeological excavations within these groups uncovered one or multiple middens (refuse sites) in each group, most of which were located between 1.12-2.3 m below modern ground surface; along with domestic refuse, these middens also contained sherds of polychrome pottery, lending evidence that these vessels were possessed by elite and non-elite, alike.[23] Furthermore, and most importantly, the largest deposit of all uncovered direct evidence of pottery production, possibly a workshop or "palace-school" near the central palace or Acropolis;[23,27] this was an important discovery, as it is rare to determine the source of production with confidence. According to Foias,[23] in order for a site to be confirmed as a production workshop, the midden must contain direct evidence of several of the following items: (1) kilns or firing pits, (2) debris left over from the production process (including ash, clumps of clay, broken ceramics and misfired or deformed sherds), (3) production tools (including molds, pot stands, polishing stones, and tools for grinding pigments), and (4) raw materials used for production (including clays,

paint pigments, and tempers). Furthermore, from all five excavation groups (A-E), roughly 20,000 sherds were uncovered, weighing approximately 1200 pounds and determined to not be mixed in with materials from earlier time periods, and so it is believed that these materials were produced on-site, rather than transported to that location.[23] This direct evidence not only lends insight into the production choices, methods, and organization of the Maya,[23] but also yields information into the nature of elite control over the production of such items.[27]

Initial results of the analysis of some of these sherd samples by instrumental neutron activation analysis[31,33] suggest that there were several varieties of paste recipes within the Ik' Style polychrome pottery, although the chemical composition of some could be linked to either the same potter/producer or a producer using the same paste recipe. This could be indicative that the center palace of Motul de San José was an elite center of production, but other workshops may have existed on the outskirts of the city; more investigation into this matter is required.[23,27,32,33]

Further chemical analyses are required of these other polychrome pottery sherds to gain more insight into the different styles and recipes used in the production processes, and to identify different workshops and how the products of these shops may have been dispersed throughout the region. Conclusions between the chemical composition of the sherds and the geographical resources available for use during the Late Classic time period might also help in pinpointing exact locations of provenance for these materials, thereby yielding information into the Maya culture and society.

5.2 Research Overview

Provenance studies of archaeological pottery sherds can help scholars gain insight into the manufacture and distribution of the artifacts, thereby assisting in drawing conclusions about inter- and intra-regional economic and political practices of ancient peoples.[23,27] While the technique of choice for chemical analyses of these samples has in the past been instrumental neutron activation analysis (INAA),[2,3,11-13] techniques involving sample dissolution and analysis by inductively coupled plasma mass spectrometry (ICP-MS) are becoming more prominent[16] with the impending decommissioning of the majority of neutron sources.

The Motul de San José Archaeological Project is a long-term, interdisciplinary, and multi-author research program, directed by Dr. Antonia Foias and Dr. Kitty Emery. Ik' Style polychrome pottery sherds from the Late Classic Period, collected between 1998 and 2005 from archaeological excavation sites within the Maya city of Motul de San José, were analyzed in this study by ICP-MS for insight into the provenance of the raw materials in order to obtain a better understanding of the economic and political structure of the Maya. This was accomplished by determining the rare earth elements (REEs), which are known to be useful for chemical fingerprinting and making definitive distinctions between pottery samples[3-6]

Validation of the determination of the REEs within the Maya polychrome pottery sherds was hindered by the inhomogeneity of the sample materials and the reference material, New Ohio Red Clay. The method of standard additions was used to limit matrix interferences, and measures were taken to lessen the extent of the heterogeneity; these included mixing the samples well, increasing the amount of sample analyzed, and

running only two-point standard addition calibrations, containing five replicates of the sample and five replicates of the sample plus a spike containing each of the REEs. The resulting data sets were statistically analyzed for outliers arising from the sample inhomogeneity, marked by departure from the average signal intensity of the other 4 replicates as determined by the Q-test. Samples were dissolved completely in nitric acid and ammonium hydrogen fluoride, thereby eliminating the need for dangerous acids, such as hydrofluoric or perchloric acid.

5.3 Experimental

5.3.1 Instrumentation

A PerkinElmer SCIEX (Ontario, Canada) ELAN 6100 plasma-source mass spectrometer was used for detection. Samples were digested in a CEM Corporation (Matthews, NC) MARSXpress microwave system, Model 230/6 with Teflon vessels. Sample solutions were filtered through Whatman Limited Puradisc 0.45 μm PES disposable filter devices (Florham Park, NJ). The efficiency of hand grinding the pottery samples was confirmed by an Omni Bead Ruptor Homogenizer (Omni International, Kennesaw, GA). Instrumental conditions and other experimental parameters are shown in Table 5.1.

5.3.2 Reagents and Sample Materials

All solutions were prepared using >18 MΩ cm deionized (DI) water from a Barnstead E-pure system (Bedford, MA). New Ohio Red Clay (NORC), purchased from the Smithsonian Institute (Washington, DC), was used as a reference material. Standards

were prepared using Multi-element Calibration Standard 2 from PerkinElmer, Inc. (Shelton, CT), containing 10 mg L^{-1} of the REEs. Certified ACS plus nitric acid trace metals grade and hydrogen peroxide (30%) were obtained from Fisher Scientific (Fairlawn, NJ). Ammonium hydrogen fluoride was purchased from Strem Chemicals (Newburyport, MA).

5.3.3 Sample Preparation

5.3.3.1 Sample Homogenization

The NORC material was a very fine powder, and so it was deemed unnecessary to grind the material further. The material was mixed well prior to sampling, however, to promote an equal distribution of REEs throughout. The pottery sherd samples, upon arrival, were also a fine, apparently uniform powder, having been previously hand-ground using a mortar and pestle after archaeological collection.

To be sure that the samples consisted of particles of uniform size, a comparison was made between the instrument signal intensities for two solutions of the sherd samples. Two portions of each sample were digested as received (i.e. without additional grinding) according to the optimized procedure described in section 5.3.3.3 (Procedure O). Two additional portions of each sample were shaken in the Omni Bead Ruptor Homogenizer with seven glass beads for approximately 1 minute, and then digested in the same manner. This produced eight solutions in total. After digestion, dilution, and filtration, the solutions were analyzed by ICP-MS and the instrument signal intensities were compared.

5.3.3.2 Moisture Content

Preliminary experiments to determine the moisture content in NORC and pottery samples were performed by measuring the amount of mass lost after heating, as a percentage of the initial sample mass. The samples were weighed and placed in an oven at 180 ºC for approximately 6-10 h, and then immediately placed inside a desiccator to cool to room temperature before remeasuring the mass. The moisture content was 1-2% for NORC and 2-5% for the pottery sherd samples tested. The moisture content was not taken into account for the final calculations for determining the concentration of REEs in the pottery sherds, however, because it was determined to have an insignificant impact on the result (on average, it only added approximately +0.1 mg kg^{-1} to the measured concentration).

5.3.3.3 Sample Dissolution

Hu et al.[22] determined that optimum digestion could be achieved when the ratio of ammonium fluoride (NH_4F) to HNO_3 was between 0.33 and 0.83 (w/v). Rather than using NH_4F, ammonium hydrogen fluoride (NH_4HF_2) was used in this study, because it is more corrosive and was therefore believed that it might be more efficient at sample dissolution. In an attempt to deal with the heterogeneity of the NORC samples, the amounts of sample material and other reagents used in this study were doubled, while still keeping the NH_4HF_2 to HNO_3 ratio within the desired limit. It was assumed that the heterogeneity of the pottery sherds would be no better that of the NORC material; according to Hu et al.,[22] "Certified reference materials are tested for their homogeneity whereas most routine samples are not and usually are coarser grained than reference

materials. Therefore, it is recommended to use sampling of no less than 50 mg as test portion size to assure representative sub sampling for routine samples." Hu et al.[22] determined experimentally that sufficient homogenization was achieved in rock powder samples using 50 mg of the material, of less than 200 mesh, while greater heterogeneity was observed for larger sample size, due to precipitation of insoluble fluorides.

As the method of standard additions was used, it was critical to weigh out sample and reagents very accurately in order to lessen the variations between vessels due to fluctuations in sample mass. According to Thompson,[46] the method of standard additions can achieve greater accuracy when multiple replicates are run at only two points along the calibration curve (i.e. multiple replicates of the unspiked sample and multiple replicates of the sample spiked at a single concentration level). For these analyses, each sample typically had a total of ten vessels: five were used as replicate blanks (containing sample but no spikes), while the other five were used as replicates for the spiked sample.

The NH_4HF_2 was first carefully ground into a fine, uniform powder in a mortar and pestle. As this material is very toxic, particularly to the lungs, grinding was performed in the hood, and a respirator was worn at all times while removing subsamples for sample preparation. Into each of the ten Teflon microwave digestion vessels, 100 ± 0.5 mg of sample, 1000 mg ± 1.0 mg of the ground NH_4HF_2, and 3 mL of concentrated HNO_3 were added. For the five spiked samples, 200 mg of Multi-element Calibration Standard 2 were added. The vessels were capped tightly and placed in the microwave for a 10 h digestion according to the program listed in Table 5.1. As the MarsExpress microwave oven can hold up to 40 samples, it was possible to run four different samples at one time. The following day, the vessels were vented slowly, the caps were removed,

and a small amount of DI water was used to wash back into the vessel any liquid that may have accumulated on the cap. Following a modified procedure of Hu et al.,[22] the contents of each vessel were poured into clean 50-100 mL beakers and any residue washed out with a small amount of DI water.

At this stage, insoluble white crystals, believed to be excess NH_4HF_2 or fluoride precipitates, could be seen. Each beaker was heated to just below boiling on a hot plate. Once all the liquid had evaporated, the beakers were removed from the heat, and while still hot, 2 mL HNO_3 were added, and the beakers were swirled to dissolve the residue. The beakers were then placed back on the hot plate and the solution evaporated to dryness a second time. Once more, the beakers were removed and an additional 2 mL HNO_3 were added with swirling to dislodge the solid material from the walls of the beakers. The contents of the beaker were poured swiftly back into the original digestion vessel from which it came, and any remaining traces of sample solution and residue were washed back into the vessels with 2.5-3 mL DI water. The vessels were capped once again and the contents digested in the microwave oven according to the same program for another 10 h. It is possible that sample was lost during the transfer from vessel to beaker and back; stainless steel bombs, as used by Hu et al.,[22] would have been more appropriate, as the vessels were heated directly on the hotplate after heating in an electric oven, without the need for transferring to glass beakers.

The following day, the vessels were carefully vented once more and any liquid found on the caps was washed back into the vessel with a small amount of DI water. The contents of each vessel were rinsed into 50 mL polypropylene tubes and diluted with DI water to 40 g ± 0.5 g, making the concentration of spikes in solution approximately 50 ng

g^{-1}. The solutions after this second digestion appeared clear and free of solid precipitates. Solutions were filtered through a 0.45 µm disposable filter disc and were then ready for analysis by ICP-MS. This optimized sample preparation procedure is later referred to as "Procedure O."

5.4 Method Development

5.4.1 Optimization of Sample Dissolution Procedure

All preliminary work involved the use of the New Ohio Red Clay reference material. As mentioned above in section 5.1.2, the validity of the concentration values for this material is not known, as the "certified values," listed in the article by Kuleff[16] are taken from a personal communication with Glascock and no further information is provided. A separate set of values, provided along with the pottery samples for this project, was obtained via a personal communication from Bishop.[47] Although the values proposed by the two communications are similar, they are not identical, and the ones given by Bishop tend to have a wider rang. The "reference" values provided by Bishop were used in this study to evaluate the accuracy of the analysis of the NORC material.

5.4.1.1 Microwave Digestion and External Calibration

Preliminary experiments involving the NORC material followed a modified procedure according to Mariet et al.[21] The benefit of this technique is that less-dangerous substances (i.e. NH_4F) can effectively be used for the digestion, rather than having to rely on hydrofluoric acid or perchloric acid; while NH_4HF_2 is considerably more dangerous than NH_4F, as a starting material, it was deemed easier to work with than HF because it is

a solid, rather than a liquid. This technique requires heating and evaporating for long periods of time, and so the procedure was, perhaps unwisely, modified to try to make it more time-efficient. For the purposes of distinguishing this method from others, it is referred to as "Procedure A" in this work.

Approximately 250 mg of the NORC material were added to a Teflon vessel containing 1 mL of an 1:1 mixture (m/v) of NH_4HF_2 and HNO_3, 1.5 mL 30% H_2O_2, and 1 mL concentrated HNO_3. A blank control vessel and seven replicates samples were prepared this way. The vessels were capped and allowed to predigest in the hood for approximately 48 h. After that, the samples were microwave digested, ramping to 180 ºC over 20 min and holding for 3 h. After cooling for about an hour, the samples were diluted to 50 g with DI water and filtered through 0.45 µm filter discs. The solutions were clear, but a white residue remained inside the vessels. An external calibration curve was prepared using dilutions of Multi-element Calibration Standard 2 in DI water. Concentrations ranged from 1 to 4000 ng g^{-1} to determine the linear range for each analyte.

It was later decided to follow the method proposed by Hu et al.,[22] but it had to be modified because of differences in available equipment. For the purposes of distinguishing this method from others, it is referred to as "Procedure B" in this work. Approximately 50 mg of NORC sample were added to the vessels, along with 500 mg NH_4HF_2 and 1 mL HNO_3. The solutions were digested at 190 ºC for 10 h. After transferring the contents of each vessel to a glass beaker, evaporating the liquid on a hotplate, adding 1 mL HNO_3, evaporating again, and adding 1.5 mL HNO_3 and 2.5 mL DI water, the solutions were returned to the appropriate Teflon vessel and microwave

digested for a final 10 h period. The resulting solutions were diluted to 25 mL and filtered.

5.4.1.2 Method of Standard Additions

Because it was difficult to matrix-match the standards to the complex matrix of the samples, and because the samples were extremely heterogeneous, the method of standard additions was also utilized. Sample (50 mg) was added to each microwave vessel together with 500 mg NH_4HF_2 and 1 mL HNO_3. To a couple of the samples, spikes ranging from 10 mg to 2 g of the Multi-element Calibration Standard 2 were added. The vessels were microwave digested for 10 h and then evaporated on the hotplate as described above in Procedure B before an additional 10 h microwave digestion, followed by dilution to 20 g DI water and filtration. For the purposes of distinguishing this method from others, it is referred to as "Procedure C" in this work. A total of five blanks and three spikes were run.

5.4.1.2.1 Optimization of Spike Concentrations

The concentration of spikes needed for each analyte was optimized by adding spikes, three replicates of each, from 40 mg to approximately 2.8 g of the Multi-element Calibration Standard 2, yielding spike concentrations between 20 and 1400 ng g^{-1} of each analyte in solution. The mass of NH_4HF_2 and NORC in each container was 500 and 50 mg, respectively, and the final solutions were diluted to 20 g after following the same procedure of microwave digesting for 10 h, evaporating, and digesting another 10 h.

5.4.1.2.2 Optimization of Sample Mass and Replicates

The mass of sample used in each digestion was also investigated. This was done with caution, as Hu et al.[22] state, "Although it is advisable to use large sample amounts to minimize or eliminate any heterogeneity in the sample powders, the possible formation of insoluble residues during decomposition procedures poses limits on sample size;" as a result, Hu et al.[22] recommended 50 mg of sample as being optimal. In an effort to make the samples more homogeneous, though, in this work, the amount of NORC sample was doubled from 50 to 100 mg, the amount of NH_4HF_2 doubled from 0.5 to 1.0 g, and the final diluted volume was also doubled to 40 g, so as not to saturate the instrument by exceeding the instrument's maximum measurable counts per second. The masses of the spikes were also adjusted such that the concentration was still 50 ng g^{-1} in solution. Given the limited supply of archaeological sample material, using sample masses greater than 100 mg was not possible. This procedure, called "Procedure D" for the purpose of clarity, also involved five replicates of the unspiked NORC material and three of the NORC with spikes. In "Procedure E," the number of replicates was increased to 8 unspiked samples and 8 spikes for better accuracy. The final, optimized procedure, referred to as "Procedure O," was identical to Procedure E, except five unspiked samples and 5 spiked samples were used.

5.4.1.2.3 Appropriateness of Correction Factors

Once the sample dissolution procedure had been optimized, it was necessary to verify that the correction factors, determined by the NORC in each run, were appropriate. This was performed by including two sets of NORC material in a single run, at ten

vessels each. The correction factor needed was determined from the first set of NORC samples, while the second set was run as an "unknown," to whose results the correction factors were applied. By comparing the measured concentrations after having applied the correction factors with the "certified" values, the correction factors could be evaluated.

5.5 Results and Discussion

5.5.1 Sample Homogenization

Table 5.2 shows the results of comparing the homogeneity of the samples (sample ID# ICP-13 and ICP-27) ground by hand versus those ground further using the Omni Bead Ruptor Homogenizer. In comparing the duplicates using the Student's t-test of two unknown sample means, all duplicate samples were significantly different from each other at the 95% confidence interval, with the exception of Yb in the ICP-13 sample after additional homogenization, although the calculated t values were overall lower for the homogenized samples by about 30%. Unfortunately, one of the replicates for sample ICP-27, ground using the homogenizer, experienced obvious sample loss, and so the solution was not run.

When the same results are compared by a paired t test, the differences between the duplicate measurements of ICP-13 (Homogenizer) and ICP-27 (hand-ground) are not statistically different at the 95% confidence, while ICP-13 (hand-ground) is. Furthermore, none of the duplicates (hand-ground versus Homogenizer) are significantly different, with the exception of ICP-13 Homogenizer duplicate 2 versus ICP-13 hand-ground duplicate 2. The paired t test, in this case, may not be suitable for making distinctions between the two data sets, because the random error in the measurements is

too large for significant differences to be seen between duplicates or samples. However, as neither t test could show significant differences between samples that were hand-ground versus those that were ground additionally with the Homogenizer, it was determined that the use of the Omni Bead Ruptor Homogenizer would not necessarily improve the homogeneity of the samples. Therefore, further homogenization was not implemented. The use of such equipment might be recommended for future studies, however; using stainless steel beads in the Omni Bead Ruptor Homogenizer might prove more efficient than the glass beads at homogenizing the pottery sherds.

5.5.2 Analysis of New Ohio Red Clay by Microwave Digestion and External Calibration

Results of using Procedure A (following the method used by Mariet et al.[21]) and Procedure B (following the work of Hu et al.[22]) are shown in Table 5.3. The table shows the "certified" values of the REEs, as well as the concentration range reported by Bishop.[47] For both techniques, the measured concentration and the percent recovery relative to the "certified" values, are shown. The recoveries are very low for all analytes, with the exception of Eu, for which the calculated concentration fell within the "certified" range of the NORC material. The reason for the low recoveries was believed to be a combination of incomplete sample dissolution and the inability of external calibration to account for the matrix effects. The extreme heterogeneity of these materials, too, was suspected as another source of error; even the certified values for the NORC, reported by Bishop[47] in Table 5.3, are widely distributed for certain elements, suggesting that this material is very heterogeneous, despite its very fine, uniform particle size. Therefore, it was decided that the method of external calibration was not well suited for this analysis.

Procedure B, based on the work of Hu et al.,[22] was chosen as the preferred digestion technique, because the resulting solutions appeared to be more thoroughly digested than those following procedure A. It is possible that, had the procedure, detailed by Mariet et al.,[21] not been modified to decrease the time needed for sample dissolution, these recoveries may have been better; the Mariet technique, in its entirety, takes about the same amount of time as the technique offered by Hu et al.[22]

5.5.3 New Ohio Red Clay by the Method of Standard Additions

The results of Procedures C-E are shown in Table 5.4, again in comparison to the "certified" values of the NORC. All three methods exhibited better recoveries than Procedures A and B, and so it was decided that the method of standard additions should be incorporated into the procedure, given the complex nature of the samples.

5.5.3.1 Optimization of Spike Concentrations

Procedure C involved spiking with three replicates ranging from 20 to 1400 ng g^{-1} of the Multi-element Calibration Standard 2. The best recoveries of NORC, relative to the "certified" values provided by Bishop,[47] were obtained when spiking at 50 ng g^{-1}. While spiking at this concentration seemed well suited for most of the analytes that were present in low quantities within the NORC material, there were problems for other analytes (such as Ce, La, Nd, and to a lesser extent, Sm) whose concentrations were higher, so spiking at 50 ng g^{-1} did not produce signals that were clearly distinguishable from those for the original sample. Figure 5.3 shows a comparison of the standard addition slopes for Ce and Yb, for which the spiked amounts were unsuccessful and

successful, respectively. Spiking at concentrations higher than that, however, tended to saturate the instrument for Ce and Tb, and poorer recoveries were observed for the other elements. Therefore, all subsequent samples were spiked with 50 ng g^{-1} of all analytes. It is believed that a correction factor can instead be applied for those more difficult analytes, since the concentrations of the spikes could not be optimized with one multi-element standard.

5.5.3.2 Optimization of Sample Mass and Replicates

The results of Procedure D are shown in Table 5.4, after doubling the sample mass and spiked amounts in an effort to limit the heterogeneity problem. The samples were diluted twice as much, though, to avoid saturating the instrument, and so the observed instrumental intensities remained the same. The results appear slightly lower than those observed for Procedure C, but it was decided that they were similar enough, and that using a larger amount of sample would be better in the long run. Therefore, a sample mass of 100 mg of sample was used throughout the remainder of the experiment.

The results of Procedure E, also shown in Table 5.4, show slightly better recoveries when the number of unspiked and spiked samples was increased to 8 replicates of each. This, too, was to limit the effects of the heterogeneity. While it would be ideal to run ten or more of each replicate in order to achieve greater accuracy, this unfortunately is not practical, given that the procedure takes three days of laborious preparation, and the microwave digestion system holds only 40 vessels. The amount of pottery sherd sample was also limited. Therefore, in order to optimize the time involved in running this analysis, while still achieving as much accuracy as possible, it was

decided that ten vessels (five samples and five samples with spikes) would be run for each sample, thereby allowing four samples to be analyzed every three day period.

With Procedures C-E, some of the measured concentrations were within the range of values specified for the NORC, while others are just outside the range given. It was decided that the low recoveries, though better than those obtained by external calibration, were not due to the inability to get the rare earth elements into solution. Instead, the low recoveries were believed to be either (a) the result of the heterogeneity of the NORC, causing the signal to be highly variable for many of the samples, or (b) the result of sample transfer loss during the evaporation stage, even though great care was taken to minimize this. As Figure 5.3 demonstrates, the heterogeneity can cause some of the samples to have higher signal intensities than the spikes. In cases when data points appeared drastically different from the other replicates, a Q-test was performed to determine whether or not that point could be eliminated as an outlier.

Also because of the issue with highly variable signal fluctuations between samples, it was deemed necessary to run ten replicates of the NORC material each time the pottery samples were run, thereby limiting each run to only three real samples at a time. This way, however, when poor recoveries for the NORC material were obtained for each run, correction factors could be applied to the NORC and, then the same correction factor could be applied to the samples, as well, yielding a rough approximation of their rare earth element concentrations. These correction factors would help make up for possible low recoveries due to sample loss during transfer stages or incomplete digestion in microwave vessels. If, after many analyses, the correction factors needed are

similar each time, then perhaps it will not be necessary to analyze the NORC with every batch.

5.5.3.3 Appropriateness of Correction Factors

The results of gauging the appropriateness of the NORC correction factors are shown in Table 5.5, following the optimized procedure, referred to as "Procedure O." Good recoveries were obtained for the majority of REEs in the first set of NORC standard additions. Good agreement is also observed between the concentration of REEs in the first set, uncorrected, and the uncorrected concentration measured in the "unknown" NORC. Table 5.5 also shows the final concentration of REEs in the "unknown" NORC, using the applied correction factor determined by the first set, as well as the percent recovery of the material in comparison with the "reference" values. The results for the "unknown" NORC are, for the most part, within the range of the "reference" values, except for La and Ce; Ce saturated the detector in the majority of the spiked samples, and both elements appeared to have such a high degree of heterogeneity such that the majority of unspiked samples gave signal intensities higher than those of the spiked samples. In these cases, correction factors are of no use when such heterogeneity is observed. With the exception of La and Ce, however, the correction factors were considered to be appropriate for accounting for sample variability within the same run.

5.5.4 Analysis of Pottery Samples by the Method of Standard Additions

During the time allotted for this work, a total of two pottery sherd samples were run, twice each, according to Procedure O. The two samples, chosen at random, were

labeled “ICP-11” and “ICP-26,” both of which were uncovered from excavation site 2A near the Acropolis (see Figure 5.2). Whereas ICP-11 was a pale orange or beige color, ICP-26 was light grey. Table 5.6 shows the results of each analysis, after removal of outliers and application of correction factors determined from the analysis of the NORC. For the first analysis, the NORC recoveries were lower than normal. The reason for this is believed to be because of slight variations in technique between the two analysts involved; only one analyst had touched the samples on the second time around that these samples were analyzed, and better recoveries were observed for the NORC. With the exception of Ce, Nd, and Sm, overall, not much variability was seen between the two replicates of the samples, and the difference in concentration of REEs between samples ICP-11 and ICP-26 was also minimal.

As was observed with the results of the sample homogenization experiment in section 5.5.1, the random error in the measurements was so large as to cause the differences between each replicate, and between each sample, to not be statistically different from each other at the 95% confidence using a paired t test.

5.6 Conclusions

Microwave-assisted acid digestion and ICP-MS were employed for the determination of rare earth elements (REEs) in Maya polychrome pottery sherds, excavated from archaeological dig sites as part of the Motul de San José Archaeological Project. The choice of sample dissolution, using nitric acid and ammonium hydrogen fluoride, proved to be safer and less expensive than traditional techniques for archaeological and geological samples that employ more dangerous acids, such as

hydrofluoric or perchloric. While the sample dissolution technique is believed to be efficient, analysis of the Maya pottery sherds was hindered by the inhomogeneity of the sample materials and "reference" material, New Ohio Red Clay. The method of standard additions was used to limit matrix interferences, and measures were taken to lessen the extent of the heterogeneity; these included decreasing particle size diameter, heating the samples in an oven prior to digestion to remove any water content, mixing the samples well, increasing the amount of sample analyzed, and running only two-point standard addition slopes, containing five replicates of the sample and five replicates of the sample, spiked to contain an additional 50 ng g^{-1} of REE standard. The resulting data sets were statistically examined for outliers (sample intensities that failed the Q-test at the 95% confidence because they were too far from the other four replicates' intensities) that arose from sample inhomogeneity or potential sample transfer loss during the solvent evaporation process. With the assumption that the samples' compositions were similar to that of the NORC material, correction factors were applied to the samples when low recoveries were obtained for the NORC.

Although considerable variation between batches of the NORC was seen in the preliminary results, later studies showed less variation as the techniques were perfected. As a large amount of sample loss is possible throughout the three-day sample dissolution procedure, it is perhaps necessary to restrict the sample handling to one analyst, alone, even though this lengthens the time of sample dissolution, greatly; this, however, would limit the amount of sample variation between operators. Furthermore, it would be beneficial to spike the samples using single-element standards, so that the more problematic analytes, such as Ce, La, and Nd, could be spiked with higher concentrations

to facilitate better distinction between the spiked and unspiked materials. Based on the good recoveries obtained for the remaining REEs within the NORC material, though, it is believed that the sample dissolution procedure, when performed as carefully as possible to eliminate sample loss, is efficient in solubilizing all of the REEs; after the second 10 h microwave digestion, the samples appeared clear and filtration was easy, as there were not a lot of particles present to clog the filter disc. It was also shown that the correction factors, determined by the NORC, can successfully be applied to other NORC samples within the same run, and so, by assuming that the archaeological sherd samples are chemically and physically similar enough to the NORC, such that the extent of sample dissolution is similar, then the same correction factors can be applied to the samples for a more appropriate estimation of the REE concentration.

It is doubtful that this procedure, as it stands, will be capable of providing useful chemical fingerprints for provenancing. The large amount of sample heterogeneity can make even aliquots of the same sherd sample appear widely different in terms of REE concentration, and so drawing definitive conclusions between different samples seems impractical. This conclusion is in accordance with several studies on archaeological provenance; for example, in a provenance study on African archaeological artifacts, Pillay and Punyadeera[7] demonstrated that, while some conclusions could be drawn, the large variability in the concentrations of some of the REEs made them "unsuitable for provenancing."

The method may have potential for further development, such as the addition of more elements to the suite of analytes. Also, when used alongside complementary techniques, such as X-ray fluorescence spectrometry, the results might allow definitive

conclusions to be drawn about sample origin, thereby serving as a suitable replacement for neutron activation analysis.

5.7 Acknowledgments

I am grateful to Dr. Antonia Foias for the opportunity to have collaborated on this project and for assistance with the sample preparation. I would also like to extend my appreciation to Dr. Hang Xiao for access to the Omni Bead Ruptor Homogenizer.

5.8 Tables and Figures

Table 5.1 Instrument conditions and other experimental parameters

Elan 6100 ICP-mass spectrometer	
RF power	1500 W
Nebulizer gas flow	1.01 L min^{-1}
Nebulizer	GemTip Cross-Flow II
Spray chamber	Scott
Detector mode	Dual mode
Sampler/skimmer cones	Nickel
Scanning mode	Peak hopping
Dwell time	100 ms per amu
Number of sweeps/reading	10
Number of reads/replicate	1
Number of replicates	5
Isotopes monitored	^{139}La, ^{140}Ce, ^{142}Nd, ^{152}Sm, ^{153}Eu, ^{159}Tb, ^{174}Yb, ^{175}Lu, ^{232}Th
MARSXpress microwave system	
Vessels	XPress vessels, 75 mL Teflon
Power	1600 W
Percent Power Operation	100%
Step 1	Ramp to 150 °C over 60 min, hold 60 min
Step 2	Ramp to 155 °C over 60 min, hold 60 min
Step 3	Ramp to 160 °C over 60 min, hold 60 min
Step 4	Ramp to 165 °C over 60 min, hold 60 min
Step 5	Ramp to 170 °C over 60 min, hold 60 min
Cool down time	>12 hours

Table 5.2 Results for the estimation of sample homogeneity in two sherd samples, ICP-13 and ICP-27. Of the four portions taken from each hand-ground sample, two were ground further using the Omni Bead Ruptor Homogenizer. After sample dissolution, sample solutions were analyzed by ICP-MS. Instrument signal intensities (listed as thousands of cps) for each sample are reported. The second portion of Homogenizer ICP-27 was lost, and so intensities are not available.

	ICP-13				ICP-27			
	Homogenizer		Hand-ground		Homogenizer		Hand-ground	
Analyte	duplicate 1	duplicate 2	duplicate 1	duplicate 2	duplicate 1	duplicate 2	duplicate 1	duplicate 2
La	18	33	21	60	32	n/a	47	15
Ce	105	193	169	334	97	n/a	270	99
Nd	30	45	32	74	18	n/a	55	22
Sm	12	15	12	24	7.0	n/a	18	9.3
Eu	6.3	7.5	6.5	11	5.2	n/a	9.9	6.0
Tb	8.3	10	8.7	18	4.4	n/a	12	7.2
Yb	2.0	2.2	21	39	12	n/a	27	18
Lu	10	11	11	20	6.1	n/a	14	9.3
Th	54	58	57	139	44	n/a	141	50

Table 5.3 Results for the analysis of New Ohio Red Clay (NORC) by Procedure A (following Mariet, et al.[21]) and Procedure B (following Hu et al.[22]). Concentrations are reported in mg kg^{-1}. The "reference" values for NORC are also given.

"Certified" values[a]				"Procedure A"		"Procedure B"	
analyte	**min conc.**	**max conc.**	**mean conc.**	**measured conc.**	**% recovery**	**measured conc.**	**% recovery**
La	52	61	**58**	2.77	5	0.467	1
Ce	100	128	**112**	12.2	11	39.5	35
Nd	29	93	**54**	4.87	9	3.49	6
Sm	7.8	11	**9.5**	1.43	15	2.87	30
Eu	1.4	2.3	**1.6**	0.558	34	1.97	120
Tb	0.14	1.6	**1.3**	0.258	21	0.0448	4
Yb	4	6.0	**4.8**	0.896	19	0.440	9
Lu	0.57	1.0	**0.77**	0.189	25	0.0420	5
Th	14	17	**16**	1.40	9	1.08	6

[a] "Certified" values provided by Bishop.[47]

Table 5.4 Results for the analysis of New Ohio Red Clay (NORC) by Procedures C-E. Specific conditions used in each procedure are listed below. Concentrations reported in mg kg^{-1}. [a] "Certified" values provided by Bishop.[47]

"Certified" values[a]				"Procedure C"		"Procedure D"		"Procedure E"	
analyte	**min conc.**	**max conc.**	**mean conc.**	**measured conc.**	**% recovery**	**measured conc.**	**% recovery**	**measured conc.**	**% recovery**
La	52	61	**58**	25.9	45	11.9	21	29.6	51
Ce	100	128	**112**	50.0	45	23.5	21	30.9	27.5
Nd	29	93	**54**	29.0	54	14.5	27	26.6	49.1
Sm	7.8	11	**9.5**	7.36	77	5.02	53	7.11	74.6
Eu	1.4	2.3	**1.6**	1.57	96	1.20	74	1.65	101
Tb	0.14	1.6	**1.3**	0.923	73	0.756	60	0.987	78.5
Yb	4	6.0	**4.8**	3.63	76	3.09	64	4.00	83.2
Lu	0.57	1.0	**0.77**	0.592	77	0.503	66	0.678	88.5
Th	14	17	**16**	14.1	90	7.01	45	11.4	72.7

Conditions used:			
amount (mg) of NORC:	50	100	100
concentration (ng g^{-1}) of spike material in solution:	50	50	50
number of NORC samples, unspiked:	5	5	8
number of spiked NORC samples:	3	3	8

Table 5.5 Correction factors determined by comparison of New Ohio Red Clay (NORC) "reference" values to measured NORC concentration, following Procedure O. The correction factors determined by the first set of NORC standard additions were applied to a second set of NORC sample, run as an unknown. Concentrations reported in mg kg^{-1}. [a] "Certified" values provided by Bishop.[47]

"Certified" values[a]				First set of NORC Standard Additions			Second set of NORC, Run as an Unknown		
analyte	**min conc.**	**max conc.**	**mean conc.**	**measured conc.**	**% recovery**	**correction factor**	**measured conc.**	**corrected conc.**	**% recovery**
La	52	61	**58**	21	37	2.7	30	81	141
Ce	100	128	**112**	46	41	2.4	215	526	469
Nd	29	93	**54**	48	89	1.1	32	35	66
Sm	7.8	11	**9.5**	8.3	87	1.2	8.0	9.2	96
Eu	1.4	2.3	**1.6**	1.8	108	0.93	1.8	1.7	102
Tb	0.14	1.6	**1.3**	0.98	78	1.3	0.98	1.3	101
Yb	4	6.0	**4.8**	3.7	77	1.3	3.8	5.0	104
Lu	0.57	1.0	**0.77**	0.60	79	1.3	0.63	0.80	104
Th	14	17	**16**	15	96	1.0	12	13	80

Table 5.6 Concentrations (mg kg^{-1}) of duplicate samples, run according to Procedure O, of two archaeological pottery sherd samples. Correction factors have been applied.

	ICP-11		ICP-26	
analyte	**replicate 1, mg kg^{-1}**	**replicate 2, mg kg^{-1}**	**replicate 1, mg kg^{-1}**	**replicate 2, mg kg^{-1}**
La	22	25	20	16
Ce	95	45	133	27
Nd	100	9.5	64	17
Sm	30	2.6	10	3.9
Eu	4.2	0.66	1.3	0.94
Tb	3.8	0.38	1.3	0.56
Yb	10	2.5	4.9	2.9
Lu	1.5	0.39	0.68	0.49
Th	10	10	6.3	11

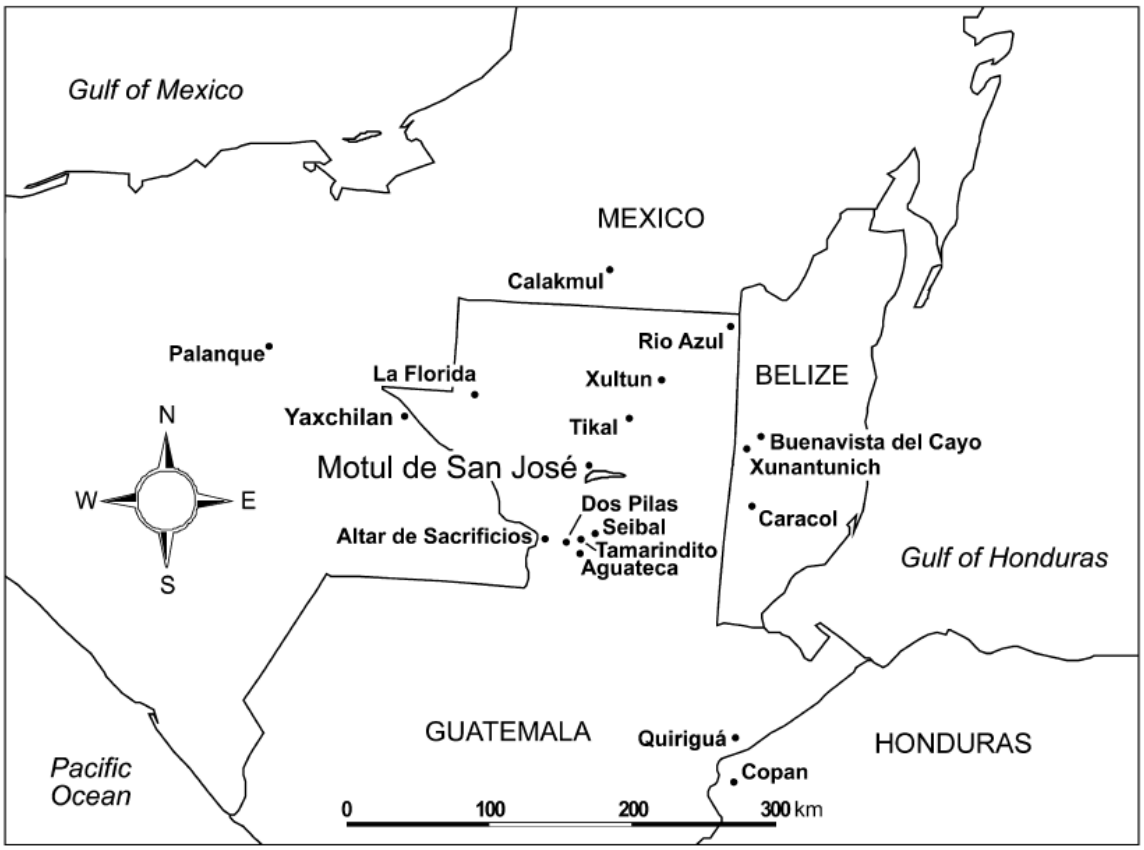

Figure 5.1 Map of Maya civilization and major Maya cities.[23]

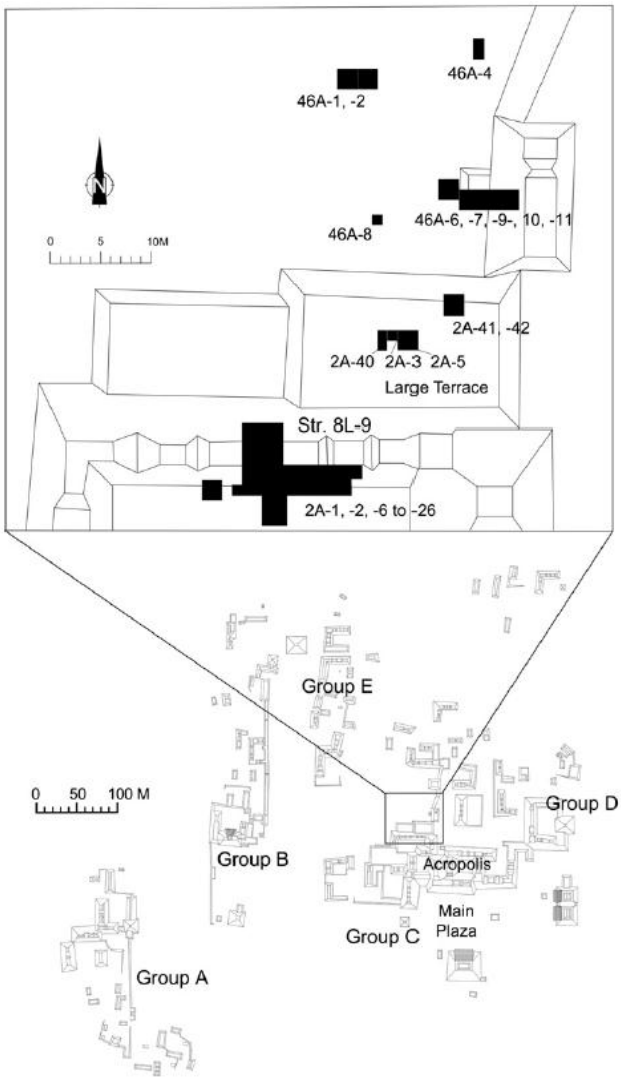

Figure 5.2 Sketch of Motul de San José excavation sites.[23]

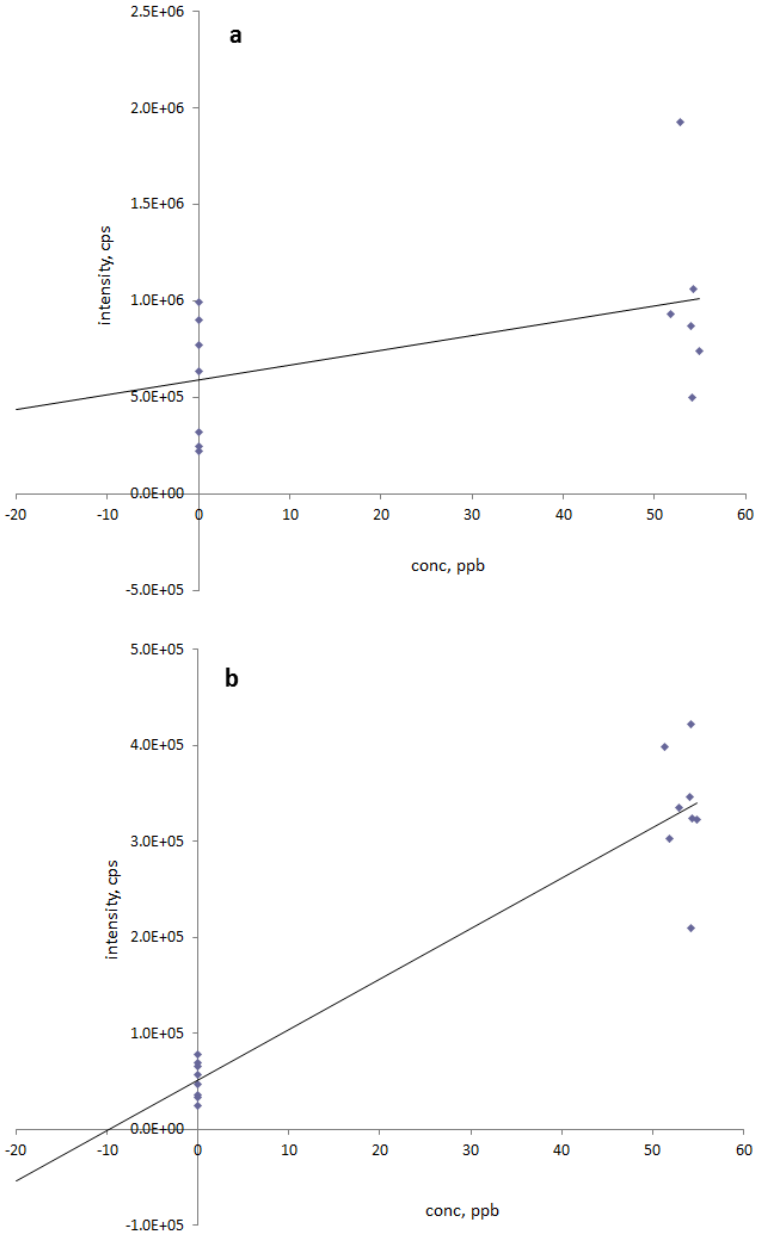

Figure 5.3 Standard additions calibration for (a) Ce and (b) Yb according to Procedure O. The greater heterogeneity, as well as the nonoptimal spike concentration for Ce is readily seen.

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CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 Conclusions

In light of today's ever-increasing demand for trace elemental determination in all areas of life, there is a need for a continuous supply of qualified analytical chemists. The improvement of current analytical techniques and the continued development of new methods are necessary to ensure that analyses can be performed with the utmost accuracy and fitness for purpose, depending on the particular problem at hand and the answers sought. As a society, we must never give up on the advancement of knowledge or the pursuit of quality. Decisions, both great and small, should be based on incontrovertible proof, supported with a degree of confidence, rather than substandard science.

6.1.1 Compound-Dependent Responses in Plasma Spectrometry

This dissertation presented evidence of compound-dependent responses in the plasma spectrometry literature, as well as experimental evidence designed to offer further proof of the phenomenon's existence. Possible explanations were explored in an effort to gain further insight into the primary cause of the differences in sensitivity. While the source of this compound-dependent phenomenon is still unknown, this work offers several ways in which the phenomenon can be eliminated, thereby limiting the extent of error introduced into ones measurements.

6.1.2 Simultaneous Determination of Inorganic and Organic Selenium Species in Dietary Supplements using Anion-Exchange HPLC-ICP-MS

A new liquid chromatographic method for the simultaneous speciation of inorganic Se and Se amino acids using isocratic elution was presented, capable of separating six selenium-containing species in under 7 minutes using a 4 mM carbonate buffer at pH 9.80 with the addition of 2% MeOH. This is the first time that dimethyldiselenide has been detected simultaneously with selenoamino acids by anion-exchange chromatography. This method also reveals that, despite being a challenge for the method, selenocystine displays very interesting behavior when mixed with the high pH of the mobile phase. Quantification was accomplished using calibration with standards and the method was applied to commercial, Se-containing dietary supplements, as well as to two reference materials, NIST 1568a rice flour and CCQM-K60 selenised wheat flour.

6.1.3 Trace Determination of Total Mercury in Rice by Conventional ICP-MS

A new method for the determination of trace concentrations of Hg by conventional ICP-MS has been developed, as well. Most studies involving Hg determination by ICP-MS involve samples that have several mg kg^{-1} of Hg, such as fish and coal samples.[1,2] Because the choice of sample dissolution technique can often lead to Hg loss,[3] and because ICP-MS is plagued by a significant Hg memory effect,[4,5] the quantitation of low concentrations of Hg is not well suited for ICP-MS. This new method is the only method capable of determining single-digit ng g^{-1} concentrations of total Hg by conventional ICP-MS and standard sample introduction systems. This method was

applied to the analysis of rice grains; L-cysteine was used to improve washout and stabilize the signals of both the mercury in solution and the gold internal standard. Two types of sample dissolution techniques, microwave-assisted acid digestion and acid extraction, were compared. The results were validated by the analysis of NIST SRM 1568a rice flour standard, as well as by a separate technique using a mercury analyzer. Furthermore, because this new method only requires a plasma-source mass spectrometer and standard sample introduction system, it could be of particular interest to labs that have only a limited need for occasional mercury determinations, and, as such, may not own a separate instrument for mercury determinations or a high-throughput sample introduction system.

6.1.4 Determination of Rare Earth Elements in Maya Pottery Sherds

Microwave-assisted acid digestion and ICP-MS were also employed for the determination of rare earth elements (REEs) in Maya polychrome pottery sherds, excavated from archaeological dig sites as part of the Motul de San José Archaeological Project. The choice of sample dissolution, using nitric acid and ammonium hydrogen fluoride, proved to be safer and less expensive than traditional sample preparation procedures for archaeological and geological samples that employ more dangerous acids, such as hydrofluoric or perchloric. While the sample dissolution technique is believed to be efficient, analysis of the Maya pottery sherds was hindered by the inhomogeneity of the sample materials and of the "reference" material, New Ohio Red Clay. The method of standard additions was used to limit matrix interferences, and measures were taken to lessen the extent of the heterogeneity; these included decreasing particle size diameter,

heating the samples in an oven prior to digestion to remove any water content, mixing the samples well, increasing the amount of sample analyzed, and running two-point standard addition slopes, containing five replicates of the sample and five replicates of the sample, spiked to contain an additional 50 ng g^{-1} of REE standard. The resulting data sets were statistically analyzed for outliers that arose from sample inhomogeneity or potential sample transfer loss during the solvent evaporation process. With the assumption that the samples' behavior during the digestion procedure was similar to that of the NORC material, correction factors were applied to the samples when low recoveries were obtained for the NORC. Although large variations were seen in the preliminary results between batches of the NORC, later studies began to demonstrate less variation as the techniques were perfected. While this procedure, at present, may not be capable of providing definitive chemical fingerprints for each pottery sherd for the study of provenance, it may demonstrate a lot of potential with further development, particularly when used alongside complementary techniques, such as X-ray fluorescence spectrometry, and it may be used eventually to replace instrumental neutron activation analysis (INAA).

6.2 Recommendations for Future Work

To ensure confidence in experimental measurements, on which to base a variety of major and minor political, environmental, and other regulatory decisions, the field of analytical chemistry should constantly be striving for the achievement of higher precision and greater accuracy while limiting error and biases as much as possible in the pursuit of quality. All this must be done in today's society while

balancing with the time and cost of analysis.

6.2.1 Importance of Sampling

In ensuring such biases are eliminated, perhaps the obvious place to start is at the beginning; the process of sampling may be the most underrated aspect of chemical analyses, and yet it is also the most important. Under no circumstances is it possible to analyze an entire sample population (although Voigtman and Abraham[6] come close); instead, enough sub-samples of the bulk material must be collected to ensure that the distribution of analytes is an accurate representation of the original material. If this initial collection stage is not performed with the utmost care, and if sufficient homogeneity of the sample material is not achieved, measurement error and other issues will accumulate further along the way, which may then mislead future decision-making.[7] Very often, samples have already been sampled and sub-sampled, prior to making it into the analysts' hands, and without proper documentation or chain of custody, the analyst unfortunately has little choice but to trust that appropriate sampling took place, and that the sample is representative of the bulk material.

6.2.2 Reliability of Standards and Reference Materials

We often take for granted that the reference standards and certified materials, that we routinely use in our laboratories to validate methods and results, can be trusted. These materials are typically certified by expert chemists from accredited laboratories, or the data are pooled from multiple laboratories in a series of round-robin experiments utilizing various techniques. Even within these "expert"

laboratories, however, substantial variation in analytical results can be obtained; for example, a recent inter-laboratory comparison was organized to investigate whether or not the determination of inorganic arsenic in rice was dependent on the method of analysis used.[8] Looking deeper into results of the study, however, the variation between the results for the determination of total arsenic was dramatic: many of the 98 participating laboratories reported concentrations, that were so far from the average, so as to be, quite literally, off the chart. This goes to show that, even for qualified, expert laboratories, trace elemental analysis and speciation can be subject to much error.

More inter- and intra-laboratory comparisons should be performed, but with stricter guidelines on which procedures to follow and how to process the data afterwards. Using the previous example again,[8] the laboratories involved in the round-robin experiment on determining arsenic in rice were given the freedom to use whichever analytical technique they preferred, although details of the individual methods chosen by each laboratory should have been included with the report. While some laboratories adjusted for moisture content, others did not; furthermore, the laboratories that did measure moisture content report ranges from 0.5-14%, suggesting that there is a need for standardized protocols to limit the variability. Finally, while the individual laboratories were told to calculate their measurement uncertainty, the individuals organizing the study report that many of these estimates were incorrect; therefore, to correct for this *uncertainty* in the *uncertainties*, the organizers appear to have, somewhat arbitrarily, applied coverage factors, which perhaps explains why some laboratories' results were so far from the average. Had

stricter guidelines been provided for these round-robin experiments, the amount of uncertainty associated with the results would have been equivalent among the different participating laboratories, thereby eliminating the need for assumptions and allowing for the data to be pooled in order to make definitive conclusions.

That being said, there is an overwhelming need for standardized methods and reliable reference and calibration standards. One example from this dissertation is the questionable composition of the sodium selenide standards purchased from both Pfaltz & Bauer and Sigma-Aldrich, which were shown to be something other than they claimed. It is suspected that the materials are actually hydrated sodium selenide and disodium diselenide, respectively, although more information is needed to confirm this. Molecular mass spectrometry, for instance, could easily provide the answer to the compounds' composition.

6.2.3 Determination of Rare Earth Elements in Maya Pottery Sherds

6.2.3.1 Sample Heterogeneity

The determination of the REEs in the Maya archaeological pottery sherds, as another example, demonstrates the fundamental problems in dealing with heterogeneous samples and in making assumptions based on similarities of those samples to another, poorly-characterized material, i.e. the NORC. More reference materials, such as granite reference materials GSR-1 and G-2, or brick clay SRM 679, would have been beneficial to this project, specifically ones that not only have been certified for their elemental composition, but also ones that have successfully been analyzed by ICP-MS;[9,10] the only analyses that have been performed on the NORC

have been by INAA, and so it is not known if other researchers, regardless of the choice of sample dissolution technique, would encounter similar low recoveries when using ICP-MS as the detector. Organizing an interlaboratory round-robin for the NORC material, in which participants are required to use ICP-MS, but are given the freedom to use a dissolution technique of their choosing, could be very effective in determining the concentration of REEs within it, as well as in detailing the extent of heterogeneity.

Another way to verify this method's ability to quantify the REEs in pottery sherds and to estimate the extent of the sample's heterogeneity is not necessarily through the analysis of standard reference materials, but rather by making a mock pottery vase: not only could the raw clay material be spiked with known concentrations of the REEs, but also, once the vase is fired, the paints used to paint the vase can be spiked with known concentrations of REEs, too. This way, the entire vase can be homogenized by thoroughly grinding and mixing, several subsamples can be dissolved and analyzed by ICP-MS, and the accuracy of the method can be verified by comparing the results to the expected concentrations of the REEs that had been added during the production of the vessel. Any heterogeneity issues discovered in the analysis of the mock vase could support the idea that the analysis of the Maya archaeological pottery sherds, too, suffers from a similar challenge.

Better recoveries might have been obtained for Ce, La, and Nd in the NORC material, had single-element standards been available for spiking, rather than relying on a multi-calibration standard; while spiking at 50 ng g^{-1} was determined to be optimal for the other REEs, this was too small of an amount to be noticeably

distinguished from the Ce, La, and Nd already present in the NORC material.

Further investigation into the means of mechanically grinding these pottery sherds would be of use, in an effort to achieve greater homogeneity. The results of the preliminary experiments using the Omni Bead Ruptor Homogenizer suggested that the samples were slightly more homogeneous than the samples that were ground by hand, but perhaps greater homogenization could have been achieved using steel beads, rather than glass ones.

This project would also benefit by a larger sample size to combat the heterogeneity. Unfortunately, due to time constraints and limited sample quantity, each pottery sherd was only analyzed twice; if more of the sample could be procured, more data could be generated for each sample, allowing for appropriate statistical analysis, such as analysis of variance (ANOVA). As it stands now, the two replicates of each sample show some similarities (after the application of the correction factor needed, determined by the recovery of the NORC material), but the random error in the measurement might be too large for any differences between the samples to be seen. For example, the measured t value, using the paired t test, would have to exceed the critical t value of 2.31 (2-tailed, 95% confidence, 8 degrees of freedom) in order for a significant difference to be observed between the REE concentrations; observing such a t test statistic, however, might be rare, as the standard deviation of the paired t values will most likely always be too large, thereby keeping the measured t test statistic below 2.31. On the other hand, using the paired t test to compare the ratios of the REE concentrations between two samples, rather than the differences, might be a more appropriate test, as the standard error would be a smaller number; however, in

doing this for the results of sample ICP-11 and ICP-26, not only are the two samples significantly different from each other, but so too are the duplicates within each sample. Even removing some of the more challenging elements from the paired t test, such as Ce, La, and Nd, does not significantly change the conclusion.

6.2.3.2 Chemical Fingerprinting

Chemical fingerprinting could be improved further by determining more analytes within these archaeological samples, besides just the REEs. The use of alternate instrumental techniques, such as X-ray fluorescence spectrometry, could also supply complementary data for better chemical mapping. This could be used to not only draw distinctions between groups of pottery sherds, but also between the sherds and the nearby geochemical surroundings to determine the origin of the artifacts and the materials used to make them, thereby providing potential information about trade among the Maya peoples.

Bishop et al.[11] made a rather pessimistic, but probably not wholly inaccurate, statement, however, about the possibility of gaining any useful information out of these kinds of analyses: "Based on [Bishop's] experience in analyzing thousands of ceramic samples from different environments around the world, "exploration" packages giving elemental determinations primarily between 10 and 30 percent probably will *not* be adequate for *intra*regional comparisons. Nor will the data be of suitable quality to store in a data bank even though acceptable standardization is carried out." As of now, this whole idea of establishing provenance based on chemical analysis seems predicated on the idea that the artifacts, as a whole, are

homogeneous; were it possible to dissolve and analyze an archaeological pottery vase, in its entirety, it is possible that the elemental composition from one side of the artifact could be widely different from a spot on the opposite side of the vase. Therefore, perhaps there is a need to reexamine the underlying hypothesis of uniform distribution of trace elements in these artifacts, and do just that: analyze an intact artifact for its trace elemental distribution. The results might suggest that a need for the reevaluation of past provenance studies is in order.

6.2.3.3 Sample Dissolution Procedure

The sample dissolution procedure used in this work, though having the advantage of using safer and less expensive reagents, was at a severe disadvantage because of the length of time required for full dissolution. The three-day process was exacerbated by the fact that only 40 samples could be run at once (a limitation of the microwave system), and at 10 replicates of each material and the need for analyzing the NORC material with every batch, only three sherd samples could be run at one time. Experiments are ongoing to analyze the remainder of the samples not covered in this work.

Hydrofluoric acid was not used in this study for a number of safety reasons. However, using HF instead of NH_4HF_2 for the sample dissolution procedure would have been more efficient in terms of length of time spent dissolving the NORC and pottery sherd samples. Even with HF, though, some researchers recommend a 12 h pre-digestion in the acid,[12] while other methods require expensive equipment to save time, such as the CEM MicroVap system used by Kennett et al.[13] Furthermore, many

methods[12,13,14] using HF also require an acid neutralization step with boric acid, which can create problems with the ICP-mass spectrometer, as it increases the total dissolved solids. More importantly, though, Mariet et al.[15] analyzed three reference materials while comparing two procedures, one using NH_4F and HNO_3, the other using the more traditional HF, HNO_3, and $HClO_4$. Although both techniques had their advantages and disadvantages, the results of both were in agreement with each other and with the certified values. A study should be performed, however, testing the efficacy of NH_4HF_2 versus NH_4F.

6.2.4 Selenium Speciation

6.2.4.1 Available Standards and Purity

The speciation of Se in dietary supplements is another topic that suffers from lack of calibration standards; this is evidenced by the presence of unknown compounds within the supplements analyzed in this study, particularly for those supplements that contain selenized yeast. Furthermore, Cuderman and Stibilj[16] demonstrated that the purity of the enzymes used for these analyses can be questionable; this has been confirmed in both this study and in others,[17] and should perhaps not be ignored when making decisions about these supplements. Preliminary tests should be performed on all lots of the enzymes used over the course of a study, in order to be aware of the potential contamination present.

6.2.4.2 Extraction Procedure

Through mass balance, the Se study also clearly demonstrated the need for a more efficient extraction method. Even the most recent procedures,[17,18] designed to combat this issue while decreasing the total extraction time, still suffer from as little as 80% recovery of extracted species. Priority should perhaps be given to improving extraction efficiency, though, and once that has been solved, then it would be appropriate to focus on how to achieve the same efficiency within a shorter time frame. Besides exploring different types of enzymes or combinations thereof, the available techniques could also be combined to achieve maximum extraction; for instance, the samples could be leached, shaken overnight in the presence of the enzyme(s), and the following day, both extraction with an ultrasonic probe and microwave-assisted digestion in the presence of additional enzyme(s) could be performed. A few researchers have had some success with lengthy extraction processes, such as Mounicou et al,[19] who extracted Se species from garlic by leaching the samples with water, extracting the species bound to the cell wall by lysing with cellulose, chitinase, protease, and β-glucanase, proteolysis with Protease XIV and Tris-HCl buffer, extraction with HCl to liberate the residual organic species, and extractions with sulfite solution and CS_2. On the other hand, multiple extractions could also be performed on the same sample material, in order to maximize the extraction.

6.2.4.3 Speciation of Reference Materials

Accurate and quantitative speciation information about foods and biological

materials is needed to better understand Se bioavailability. This should start, first and foremost, with a reference material with known speciation data; for instance, the NIST 1568a rice flour, which has been certified for its total Se content, should be certified for its Se species content, as well, so that researchers can gauge the accuracy of their proposed methods and techniques. Although the method used in this work was not capable of speciation of this material, as the total concentration was too close to the method detection limit, it is possible that multiple Se compounds were present, in addition to the large amount of selenomethionine and/or seleno-(methylseleno)cysteine.

Modifications to this method could be implemented to better understand the species content of the rice flour material. For instance, using a different extraction procedure, that is capable of using more than 200 mg of sample (a drawback of the 10kDa molecular weight cut-off filter), could help increase the amount of Se within each sample solution, thereby making it easier to distinguish from the detection limit. Using a larger injection loop volume, a shorter column length, or even a different sample introduction system, too, could improve the detection limits of this method. The Scott spray chamber used in this study is not well suited for speciation analysis, as it has lower aerosol transport efficiency and more noise compared to typical cyclonic spray chambers;[20] miniature nebulizers and spray chambers have also been shown to offer some of the best signal to noise ratios.[21] Using an ICP-mass spectrometer, equipped with a dynamic reaction/collision cell, too, would help improve the DLs by lowering the background noise; that way, too, other Se isotopes, besides m/z 78.96, could be monitored.

6.2.4.4 Detection Limits

While on the subject of detection limits (DLs), there is also a need for a more standardized way of reporting DLs in liquid chromatography. A survey of the recent literature reveals that researchers report their DLs in a variety of different ways, many of which might be chosen because it makes their methods appear better on paper, but are not completely indicative of the true detection capability of the method. A standard protocol for calculating detection limits in HPLC should be implemented, based on peak area, rather than peak height, for greater accuracy. For instance, the method used in Chapter 3 of this dissertation verified the appropriateness of the calculated DLs by visually inspecting the chromatograms of a single standard that was continuously diluted by half until the chromatographic peaks could no longer be distinguished. If all researchers reported DLs in the same way, then DLs could easily be compared across methods, rather than having to decipher first how the calculation was made.

The appropriate method of calculating true DLs, too, rather than falsely assuming it to be three times the standard deviation of the blank concentration, has recently been re-evaluated to take into account the Currie decision levels, probabilities of false positives and negatives, and correct treatment of the noise, among other characteristics of the measurement;[22,23] while it may be difficult to persuade researchers to abandon the equation they have been using for decades, this revised method of calculation should be implemented in future research.

6.2.4.5 Role of Selenium in Human Health

Speciation of selenium in foods, dietary supplements, and biological materials, to better assess the bioavailability in humans, is a topic that demands a lot more attention. With the conflicting results of the Nutritional Prevention of Cancer (NPC) Trial and the Selenium and Vitamin E Cancer Prevention Trial (SELECT), as well as the tragic death of 21 polo horses[24] in 2009, due to accidental selenium overdose, selenium has acquired a tarnished reputation in the media and the general population is perhaps equally conflicted. After the failure of SELECT, there was a marked disappearance of selenium-containing dietary supplements from local drugstores and pharmacies. Now, though, as more evidence of the benefits and anti-carcinogenic properties of certain selenium compounds emerge, "selenium" is making a reappearance on the shelves.

However, experts argue that caution should be exercised; for example, according to Stranges et al.,[25] "Additional experimental evidence is needed to provide new insights into the role of selenium and of specific selenoproteins in human biology, especially to clarify the underlying mechanisms linking selenium to chronic disease endpoints . . . This would help determine the optimal level of selenium intake in the general population that can maximize health benefits while avoiding potential chronic toxic effects." These additional experiments should compile data spanning multiple techniques, including anion-exchange chromatography with ICP-MS, as was used in this work, as well as cation-exchange, reversed-phase, or even size-exclusion chromatographies, and more importantly, molecular mass spectrometric techniques; all of these, combined, have the capability of unambiguous species identification and quantification.[26] Modification to

the current, recommended daily allowance of Se (55 μg day^{-1} for adults[27]) should perhaps reflect the chemical form of Se.

6.2.4.6 Regulations on Dietary Supplements

Finally, while it is unlikely that the Food and Drug Administration (FDA) will regulate dietary supplements in the near future, perhaps the advent of faster and less expensive screening capabilities might help individual manufacturers routinely police their products so that more accurate labeling may be achieved.

6.2.5 Mercury Determination

6.2.5.1 Mercury Speciation

Besides Se speciation, species determination is also needed for the determination of mercury found in rice. While the rice samples analyzed in this work contained only small quantities of total Hg (approximately 3-6 ng g^{-1}), this small amount could actually be more problematic in terms of long-term effects if it turns out that the majority of the Hg is present in the methylated form. As Hg can accumulate in the body over time, and because rice is a staple food in many regions of the world, the effects could be worrisome, particularly for children and developing fetuses. Studies show, for example, that methylmercury is readily incorporated into rice grains that were grown near Hg mining districts in China.[28] The heterogeneity of rice grains, however, is also something that warrants further investigation. Analysis of the grains within an entire bag of rice might be necessary, in order to better estimate the distribution of Hg, or other trace elements such as Se and As, throughout each rice

grain, although it may still be difficult to draw definitive conclusions between one bag of rice and another.

Speciation studies of Hg in rice could, in part, be accomplished by modifying the anion-exchange chromatographic technique used for Se speciation in this work, to separate the different Hg compounds; appropriate standards, such as methylmercury and mercury salts, would be needed for calibration, and optimization of the mobile phase composition and pH would be required, as Hg compounds have different pK_a values than Se compounds. However, a much more sensitive means of detection would be required for the detection of Hg species in rice grains. Selenium speciation in the NIST SRM 1568a rice flour material was met with difficulty in this study because the concentration in solution was too close to the method detection limit; furthermore, whereas the rice flour SRM contains 380 ng g^{-1} Se, it contains even less Hg (5.8 ng g^{-1}), and so the method, as it stands, would not be able to detect any Hg, at all. Coupling the separation to other techniques, such as atomic fluorescence spectrometry, might be an option worth exploring.

6.2.5.2 Mercury Surveillance in Food

There is a growing need for the FDA to set regulations of Hg contamination in a variety of foods, as currently their primary surveillance focuses on Hg in fish.[29,30] Furthermore, more sophisticated methods, such as the one discussed in this work but capable of determining trace concentrations of the toxin with greater accuracy and precision, are needed for routine screening of other foodstuffs in order to assess the potential health risks. While instrumentation exists, such as CVAAS and mercury

analyzers, for the specific determination of Hg, it would be beneficial to have more multi-element techniques that are also capable of accurate Hg determinations, so that more laboratories could screen foodstuffs for it. These could include the detection of single-digit ng g^{-1} concentrations of Hg by ICP-MS, ETAAS, and AFS, to name a few.

6.2.6 Compound-Dependent Responses

One final remark about analytical quality emerges from the example of compound-dependent responses in plasma spectrometry. Plasma spectrometry is supposed to be a technique that is independent of an element's chemical form, but at least for arsenic and selenium, this is not always the case. While the specific cause of the phenomenon is still at large, and more experiments should be devised to test for hydride formation, loss to lateral diffusion, or potential differences in the mechanisms of desolvation/vaporization/atomization processes, raising awareness of this strange occurrence seems the first step in correcting for it. To test for some of these other theories, future experiments could perhaps make use of flow injection with hydride generation, high speed cameras, dynamic light scattering, or even transmission electron microscopy in an effort to sight potential differences between the species' droplets as they emerge from the nebulizer, and their fates afterwards; perhaps also there is a way to set up sensors within the torch box of the ICP-emission spectrometer, to monitor hydride species or species that may be lost to lateral diffusion. It has been demonstrated in this work that ignorance of, or even complete disregard of the phenomenon, can result in measurement bias when a variety of

inorganic and organic Se species are concerned. Steps, such as the advice offered at the end of the second chapter of this dissertation, should be taken to eliminate the occurrence of this phenomenon whenever possible to ensure greater accuracy.

6.2.7 Conclusion

In conclusion, analytical chemists, charged with the job of seeking quality in analytical measurements, are faced with a number of challenges. Although they may be concerned with how confidently they can make accurate quantifications, they perhaps may also need to look deeper into factors that are quite often taken for granted; these include the assumption that the available reference materials and calibration standards are accurate, that the reagents and enzymes being used are uncontaminated and pure, that their instrumental techniques are capable of reporting unbiased measurements that are *independent* of an analyte's chemical structure, and that their sample materials have undergone appropriate sampling, are homogeneous in composition, and are representative of the bulk material. Awareness of the possible sources of measurement bias and error is the first step in accounting for them, and the first step in approaching quality of measurement.

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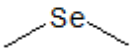
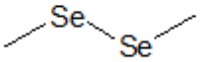
APPENDIX

RELEVANT SELENIUM COMPOUNDS AND THEIR STRUCTURES

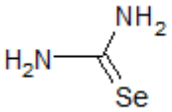
dimethylselenide (DMSe)[1]



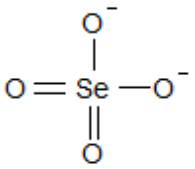
dimethyldiselenide (DMDSe)[1]



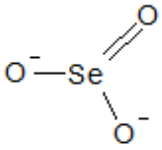
selenourea[1]



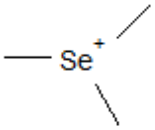
selenate (Se^{VI})[1]



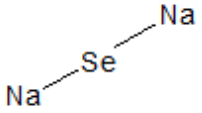
selenite (Se^{IV})[1]



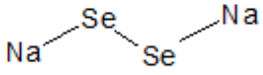
trimethylselenonium ion[1]



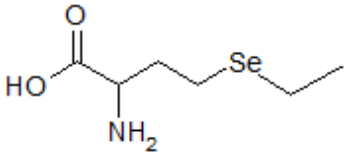
sodium selenide (Na_2Se)[2]



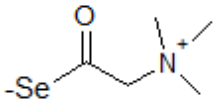
disodium diselenide (Na_2Se_2)[3]



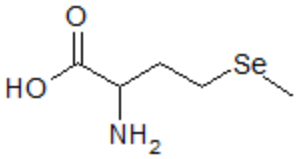
selenoethionine[4]



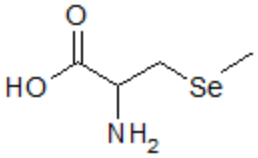
selenobetaine[1]



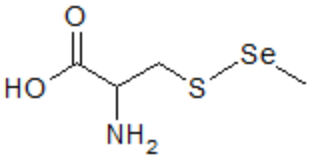
selenomethionine (SeMet)[1]



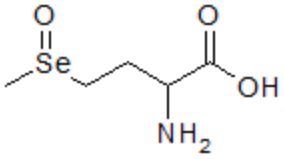
Se-(methylseleno)cysteine (SMSC)[1]



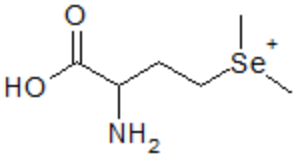
S-(methylselenocysteine)[5]



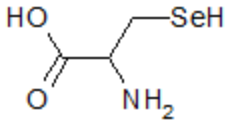
selenomethionine selenoxide[2]



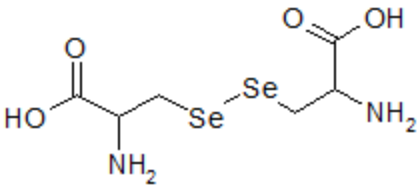
Se-(methylseleno)methionine[1]



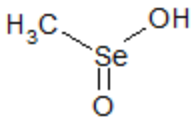
selenocystine[1]



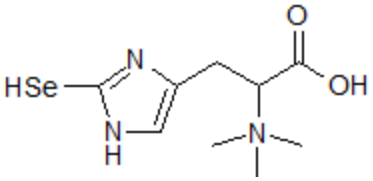
selenocysteine [$(SeCys)_2$][1]



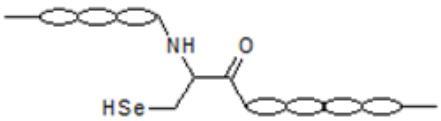
methylseleninic acid[2]



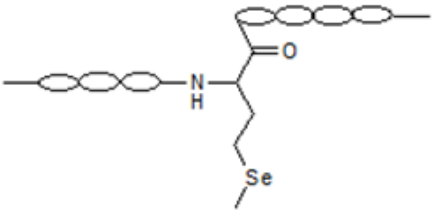
selenoneine[6]



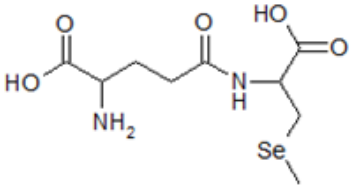
selenoprotein
(Se incorporated into protein as selenocystine)[1]



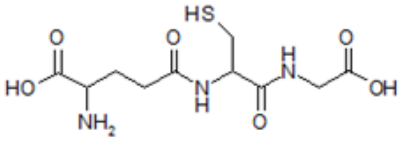
selenium-containing protein
(Se incorporated nonspecifically as SeMet)[1]



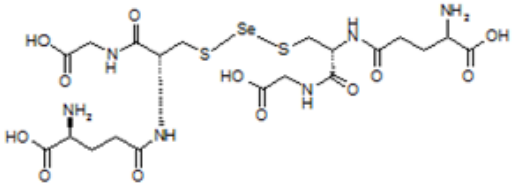
gamma-glutamyl-seleno-(methylseleno)cysteine[1]



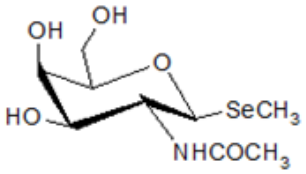
glutathione[6]



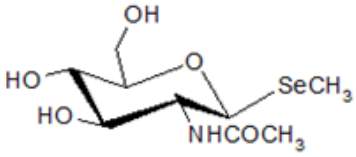
selenodiglutathione[7]



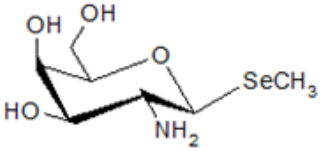
"selenosugar 1" (methyl-2-acetamido-2-deoxy-1-seleno-β-D-galactopyranoside or Se-methyl-N-acetylgalactosamine)[8]



"selenosugar 2" (methyl-2-acetamido-2-deoxy-1-seleno-β-D-glucopyranoside or Se-methyl-N-acetylglucosamine)[8]



"selenosugar 3" (methyl-2-amino-2-deoxy-1-seleno-β-D-galactopyranoside)[8]



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